University of Alberta

Optimizing impermeant support in an intraluminal preservation solution tailored to the small intestine.

by

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Abstract

The AA (amino acid) solution is an experimental preservation solution delivered intraluminally and tailored to the metabolic requirements of the small bowel. Preservation solutions contain impermeant molecules to prevent edema formation. Dextrans and hydroxyethylstarches are two such oncotic agents. The effectiveness of these molecules has never been compared, nor has the appropriate concentration ever been established within the AA solution. Rat intestines were harvested after an intravascular flush with University of Wisconsin (UW) solution and flushed intraluminally with UW or AA solution containing either dextran70 (D70; MW= 70 kDa) or hydroxyethylstarch (HES; MW= 2,200 kDa). Fluorescent microscopy, energetics, oxidative stress, and histology revealed the superiority of the HES-supplemented AA solution. In a subsequent experiment, tissues were preserved with AA solution containing 0, 2.5, 5, or 10% HES. Energy parameters and histology were superior in the 5% group. Together, these studies support the use of 5% HES within the AA solution.

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Abbreviations

adenosine diphosphate = ADP

adenosine monophosphate = AMP

adenosine monophosphate – activated protein kinase = AMPK

adenosine triphosphate = ATP

albumin – dextran – adenosine – allopurinol – verapamil = ADAAV

amino acid = AA

analysis of variance = ANOVA

anterior inferior pancreaticoduodenal artery = AIPD

antineutrophil serum = ANS

bicarbonate = HCO_3^-

N,N – Bis (2 – hydroxyethyl 1) taurine) = BES

chloride = Cl^-

cholecystokinin = CCK

colic branches = COL

cyclic adenosine monophosphate = cAMP

dalton = Da

dextran 70 = D70

4',6-diamidino-2-phenylindole = DAPI

energy charge = EC

ethylenediaminetetraacetic acid = EDTA

eurocollins = EC

extracellular fluid = ECF

extracellular-signal-regulated kinase = ERK

fatty acid = FA

fluorescein 5 – isothiocyanate = FITC

gasterointestinal = GI

glucagon – like peptide = GLP

glutathione = GSH

glutathione disulphide = GSSH

graft versus host disease = GVHD

gram = g

glucose transporter = GLUT

hematoxylin and eosin = H&E

histidine tryptophan ketoglutarate = HTK

home parenteral nutrition = HPN

hydrogen = H^+

hydrogen peroxide = H_2O_2

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid = HEPES

hydroxyethylstarch - HES

hydroxyl radical = ·OH

hypochlorous acid = HOCl

hypoxanthine = HX

integrin beta-2 = CD18

ileal branches = IL

ileocolic artery = IC

iron = Fe

ischemia – reperfusion = IR

jejunal branches = JEJ

c-Jun N-terminal kinase = JNK

killodalton = kDa

kilogram = kg

krebs – henseleit – bicarbonate buffer = KHB

lactated ringer’s = LR

leukotriene B4 = LTB_4

middle colic artery = MC

millimolar = mM

milliosmoles = mOsm

P38 mitogen-activated protein kinase = P38

molar = M

myeloperoxidase = MPO

nanomol = nmol

nicotinamide adenine dinucleotide phosphate (reduced) = NADPH

nicotinamide adenine dinucleotide (reduced) = NADH

nitric oxide = NO

N^6 – nitro – L – arginine, methylester = L – NAME

nuclear factor-kappaB = NF - kB

number average molecular weight = MW_N

organ procurement and transplantation network = OPTN

osmotic reflection coefficient = σ_D

oxygen = O_2

oxygen free radical = OFR

pancreatic polypeptide = PP

parenteral nutrition = PN

parenteral nutrition associated liver disease = PNALD

peptide transporter = PepT

peptide tyrosine tyrosine = PYY

perfluoro chemical = PFC

phosphate buffered sucrose = PBS

platelet activating factor = PAF

polydispersity index = PDI

polyethylene glycol = PEG

posterior inferior pancreaticoduodenal artery = PIPD

potassium = K^+

potassium chloride = KCl

reactive oxygen species = ROS

red blood cell = RBC

right colic artery = RC

scientific registry of transplant recipients = SRTR

short bowel syndrome = SBS

small bowel transplantation = SBTx

sodium = Na^+

sodium – glucose cotransporter = SGLT

standard error = SE

student-newman-keuls = SNK

superior mesenteric artery = SMA

superoxide = $\cdot O_2$

superoxide dismutase = SOD

total adenylate = TA

triglyceride = TG

university of wisconsin = UW

vasoactice intestinal polypeptide = VIP

weight average molecular weight = MW_w

williams medium E = WME

xanthine dehydrogenase = XD

xanthine oxidase = XO

Chapter 1:

Anatomy, Physiology, Transplantation, Ischemia/Reperfusion Injury, and Preservation of the Small Intestine

Gross Anatomy (1)

The small intestine resides within the abdominal cavity, inferior to the stomach and the liver. Measuring about 2.5cm in diameter and 2.7 to 4.5m in length, the highly convoluted small intestine is the longest part of the gastrointestinal tract. Three regions comprise the small intestine: the duodenum, jejunum, and ileum.

The duodenum is the first 25cm segment. Receiving the stomach contents, pancreatic juice, and bile, a number of processes occur here; bile acid emulsifies fats, stomach acid is neutralized, the high pH inactivates pepsin, and pancreatic enzymes continue chemical digestion. Starting at the pyloric valve, the duodenum wraps around the head of the pancreas and continues leftward, terminating at a bend known as the duodenojejunal flexure. Most of the duodenum is retroperitoneal. Near the pyloric valve, in the most proximal portion of the small intestine, small wrinkles called the major and minor duodenal papillae are seen. This is the point of insertion for the pancreatic duct and the accessory pancreatic duct, respectively.

Digestion and nutrient absorption primarily occurs in the jejunum. For the most part, it lies within the umbilical region; however, it truly begins in the upper left quadrant of the abdomen. Technically, the jejunum is the first 40% of the small intestine

starting after the duodenum, or roughly 1.0 to 1.7m. The jejunum is characterized by tall, large, tightly packed circular folds, with a thick, muscular wall and a rich blood supply.

The hypogastric region and part of the pelvic cavity is where we find the ileum. It forms the distal 60%, or about 1.6 to 2.7m, of the post-duodenal small intestine. The ileum, as compared to the jejunum, has a thinner, less muscular wall, and is less vascularised. Aggregated lymphatic nodules called Peyer patches are visible on the surface of the ileum that is opposite to where it attaches to the mesentery. These patches progressively increase in size towards the large intestine.

Forming the ileocecal junction, the end of the ileum meets with the cecum of the large intestine. The ileocecal valve is formed at this point by muscle layer thickening of the ileum into a sphincter. This valve regulates the flow of intestinal contents from the small to large intestine. Being intraperitoneal structures, both the jejunum and ileum are draped with a serosa that is continuous with the mesentery, which suspends the small intestine from the posterior abdominal wall.

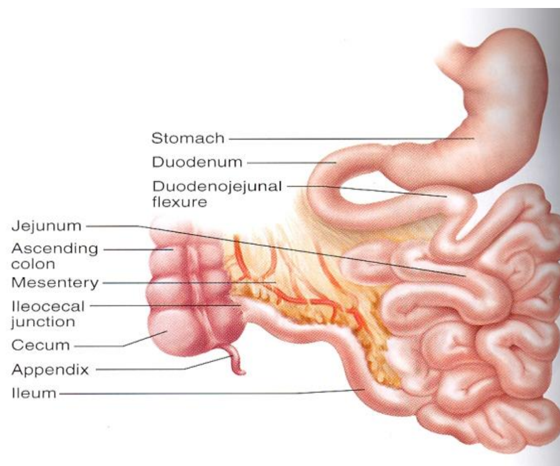

Figure 1-1. Gross anatomy of the small intestine. (1)

General Microscopic Anatomy (2)

Like the large intestine, the small intestine is made of four layers: mucosa, submucosa, muscularis, and adventitia (Figure 1-2).

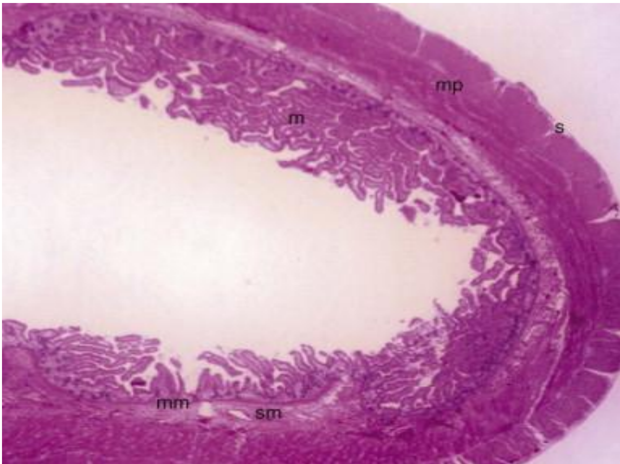

Figure 1-2. Photomicrograph of the small intestine showing its general microscopic architecture. m, mucosa; mm, muscularis mucosae; mp, muscularis propria; s, serosa; sm, submucosa. (Hematoxylin and eosin, ×25.) (2)

Mucosa

Glandular epithelium, lamina propria, and muscularis mucosae form the innermost layer, termed the mucosa. Supporting the epithelium, the lamina propria is a busy area of reticular connective tissue. Elastin, reticulin, and collagen fibers are found here, as well as lymphocytes, plasma cells, eosinophilic granulocytes, and lymphatics and capillaries. The glandular epithelium forms cylindrical units called crypts. This epithelium also contains a variety of cell types, including absorptive cells (also known as columnar cells, stem cells), undifferentiated crypt cells, M cells, and secretory cells (Paneth cells, goblet cells, and enteroendocrine cells). At the mucosa and submucosa boarder, the lamina propria is a thin layer of smooth muscle.

Stem cells lie at the base of the intestinal crypts. These cells are pluripotent, meaning that they can give rise to any type of mature intestinal epithelial cells. The absorptive cells have a brush boarder that is composed of microvilli, which increase the small bowel enterocyte surface area 14-40 fold. Goblet cells are oval or round, and have flattened basal nuclei. The broad based, flask shaped paneth cells contain antimicrobial peptides, zinc, growth factors, and secrete lysoenzymes.

Enteroendocrine cells are highly specialized cells that have a specific endocrine function. These basally located cells are oval or triangular, and have a pale cytoplasm filled with dark-staining granules. There are a variety of enteroendocrine cell types identified by electron microscopy and immunohistochemistry. Serotonin, vasoactive intestinal polypeptide (VIP), and somatostatin D cells are found throughout the small intestine, as well as the large intestine. Other cell types are found primarily in the proximal small intestine and the stomach, including gastrin-, ghrelin-, gastric inhibitory peptide (GIP)-, secretin-, and cholecystokinin-producting cells. The distribution of peptide YY-, glucagon-like peptide (GLP)-1-, GLP-2-, and neurotensin-secreting cells remain largely confined to the ileum. Motilin is mainly found in the duodenum and jejunum. In addition to releasing hormones in the blood, neuroendocrine cells also play an important role in regulating secretion, absorption, motility, mucosal cell proliferation, and potentially immunobarrier control.

M cells overlie lymphoid follicles in the small bowel and colon. These specialized epithelial cells selectively bind, process, and present pathogens to components of the mucosal lymphoid system, such as lymphocytes and macrophages.

Regarded as the pacemaker cells of the intestine, the interstitial cells of Cajal (ICC) generate slow waves and determine the frequency of smooth muscle contraction

to regulate intestinal motility. They are mesechymal cells located in both the small and large intestine that are spindle-shaped, with large ramified extensions, and contain large, oval, light-staining nuclei. The ICC also set the smooth muscle membrane potential gradient, mediate neuronal signals from enteric motor neurons to smooth muscle cells, and amplify neuronal signals. The number of ICC is regulated by serotonin.

Submucosa

The submucosa is a layer of fibrous connective tissue that sits between the muscularis mucosae and the muscularis propria. It houses fibroblasts, mast cells, blood and lymph vessels, and a nerve fibre plexus (Meissner's plexus) made of non-myelinated, postganglionic sympathetic fibres, and parasympathetic ganglion cells.

Muscularis

Being primarily responsible for contractility, the muscularis consists of two smooth muscle layers. Between the inner circular layer and the outer longitudinal layer is where the myenteric plexus is located. Parasympathetic and postganglionic sympathetic fibres terminate in parasympathetic ganglion cells, and postganglionic parasympathetic fibres terminate in smooth muscle.

Adventitia

The outermost layer of connective tissue is called the adventitia. However, when a single layer of mesothelial cells covers the adventitia, it is called serosa.

Specific Microscopic Anatomy (2)

Mucosal folds and villi characterize the small intestinal mucosa. The folds are made of mucosa and submucosa. Villi are mucosal folds that decrease in size from the proximal to the distal small intestine. Also, they have different shapes depending on their location. In the duodenum they may be broad, short, or leaf-like, while those in the jejunum are more tongue-like. Moving distally from the jejunum, villi become more finger-like (Figure 1-4A). Normal villus height is 0.5 to 1.5mm, and they are lined by enterocytes, goblet cells, and enteroendocrine cells.

Burner's glands and crypts of Lieberkühn are the two gland types of the small intestine (Figure 1-3 and 1-4B). The former secrete a bicarbonate-rich alkaline secretion, helping to neutralize gastric chime. They are mostly found in the proximal duodenum. Intestinal crypts are tubular glands that reach the muscularis mucosa. The cells that predominate here are undifferentiated cells and Paneth cells (Figure 1-3). Migration occurs as cells are generated at the crypt base and move toward the villus tip. During this phenomenon, cells mature and differentiate into a secretory lineage (goblet cells, enteroendocrine cells, Paneth cells) and enterocytes. Stem cells lose their ability to divide in the upper third of the crypt, and are thus committed to differentiate at this level.

The cryptal base contains mainly Paneth and columnar cells (Figure 1-3. Higher from the base are absorptive cells, and oligomucin cells that differentiate into goblet cells. The upper half of the crypt contains many goblet cells interspersed with enteroendocrine cells. CD3+ intraepithelial T lymphocytes are housed in the villi. Smooth muscle in the small intestinal villus is found in the lamina propria, where plasma cells and mast cells are also found. Seen in the lamina propria and the submucosa,

lymphoid tissue presents as solitary nodules and as confluent masses called Peyer's patches.

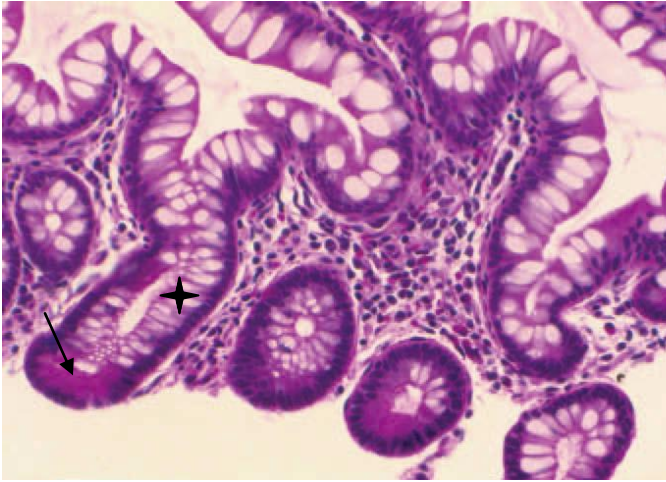

Figure 1-3. Photomicrograph of the small intestinal mucosa demonstrating the crypts of Lieberkühn (star) and Paneth cells (arrow). (Hematoxylin and eosin, ×250) (2)

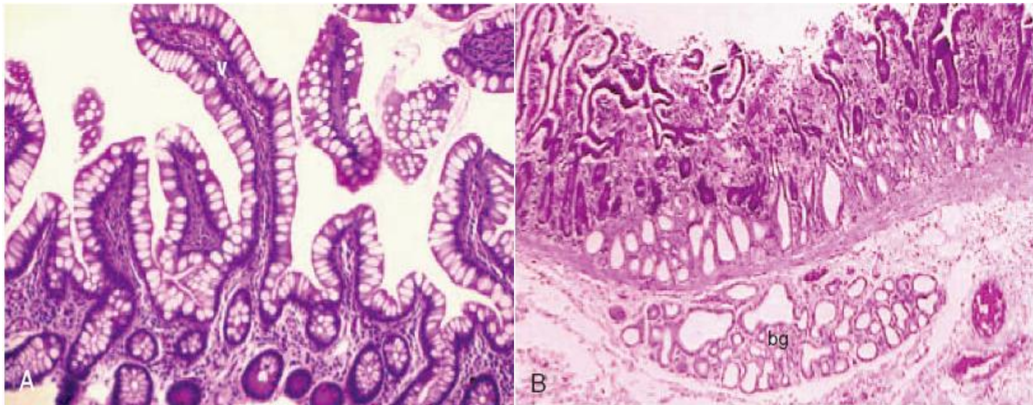

Figure 1-4. Photomicrograph of the duodenal mucosa. A*,* Villi are seen as finger-like projections. B*,* Brunner glands (bg) are found below the mucosa. (Hematoxylin and eosin. *A,* ×250; *B,* ×150) (2)

Vasculature

The distal duodenum, jejunum, and ileum receive oxygenated blood from the superior mesenteric artery (SMA). Five major vessels branch from the SMA, each supplying their named section of the small intestine: the anterior and posterior inferior pancreaticoduodenal vessels, middle colic, right colic, and ileocolic arteries. There is also

a series of jejunal and ileal branches extending from the SMA. A series of arcades form the intestinal branches, and numerous straight vessels entering the intestinal wall arise from the terminal arcade (Figure 1-5). The remainder of the duodenum is supplied by two branches of the common hepatic artery: the gastroduodenal and anterior superior pancreaticoduodenal arteries. (3)

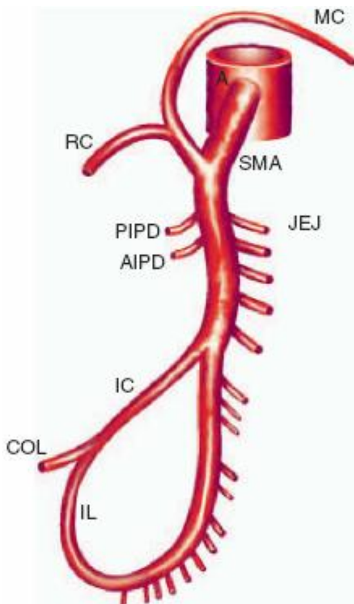

Figure 1-5. Diagram of typical superior mesenteric artery (SMA) anatomy. AIPD, anterior inferior pancreaticoduodenal artery; COL, colic branches; IL, ileal branches; IC, ileocolic artery; JEJ, jejunal branches; MC, middle colic artery; PIPD, posterior inferior pancreaticoduodenal artery; RC, right colic artery. (3)

Large plexuses from once large arterial branches enter the muscularis and pass into the submucosa. Some plexus arteries branch on the inner surface of the muscularis mucosae and divide into a network of capillaries that surrounds the intestinal crypts. Other arteries travel to the intestinal villi where they enter at the base and form a dense collection of capillaries below the epithelium of the villis (2).

Along with blood from the pancreas and spleen, blood from both the small and large intestine drains via the hepatic portal vein to the liver. From the liver, the hepatic veins bring the blood to the inferior vena cava (4).

Lymphatic Drainage (2)

Lacteals are the lymphatics of the small intestine. With the exception of the duodenum, where multiple lacteals may exist, each villus has one central lacteal. The lymphatic drainage of the small bowel parallels the blood supply to lymph nodes in the celiac, superior pre-aortic, and inferior pre-aortic regions. Lymph drainage continues to the cisterna chyli and then into the left subclavian vein, by way of the thoracic duct.

Digestion and Absorption (5)

Carbohydrates

Polysaccharides, disaccharides, and monosaccharides are the primary carbohydrates consumed in the human diet. In terms of polysaccharides, only starches (glucose polymers) and their derivatives are digested by our gastrointestinal system. Amylopectin constitutes the vast majority of our dietary starch. Unlike amylose, which is a straight chain of glucose molecules connected by only 1:4α linkages, amylopectin is a branched molecule. Ingested disaccharides are lactose and sucrose. Fructose and glucose are monosaccharides.

Although digestion of starches begins with salivary α-amylase in the mouth, the ideal pH for this enzyme is 6.7, so its action is inhibited once food enters the acidic environment of the stomach. However, in the small intestine 1:4α linkages are hydrolyzed by both salivary and pancreatic α-amylases. These enzymes leave 1:6α linkages and terminal 1:4α linkages intact, resulting in oligosaccharides as the end products of α-amylase digestion. Three oligosaccharides are produced: the disaccharide

maltose, the trisaccharide maltotriose, and α-limit dextrins, which are glucose polymers, containing about eight molecules with 1:6α linkages.

Enzymes, called oligosaccharides, that further degrade the starch derivatives, are found in the brush boarder of small intestinal epithelial cells. Hydrolysis of 1:6α linkages largely occurs through the action of isomaltase. This enzyme also degrades maltose and maltotriose, with the help of maltase and sucrase. Pancreatic proteases are responsible for the hydrolysis of the single glycoprotein chain that is inserted into the brush boarder membrane, yielding sucrase and isomaltase.

Sucrose is hydrolysed into a molecule of glucose and a molecule of fructose by sucrase. The brush boarder contains two disaccharidases. Trehalase hydrolyzes trehalose, a 1:1α dimmer of glucose, into two glucose molecules.

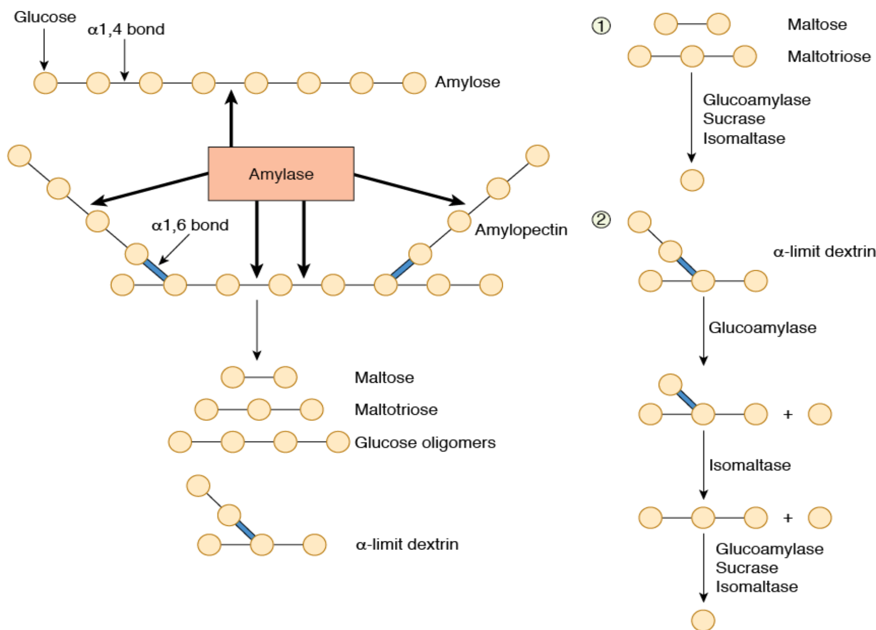

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Figure 1-6. Left: Structure of amylose and amylopectin, which are polymers of glucose (circles). These molecules are partially digested by the enzyme amylase, yielding the products shown at the bottom of the figure. Right: Brush border hydrolases responsible for the sequential digestion of the products of luminal starch digestion (1, linear oligomers; 2, alpha-limit dextrins). (5)

Hexose sugars are readily absorbed by mucosal cells in the small intestine, passing into capillary blood, which drains into the portal vein. The sodium-dependent glucose transporter is the Na^+ and glucose cotransporter responsible for transport of glucose and galactose within the small intestine (Figure 1-7). Like the glucose transporter (GLUT) series of transporters, members of this transporter family (SGLT 1 and SGLT 2) transverse the cell membrane 12 times and have their -COOH and -NH_2 terminals on the cytoplasmic side of the membrane. The SGLT-1 transports glucose and galactose in the gut, and depends on the Na^+ concentration within the intestinal lumen. Epithelial cell sugar influx is facilitated and inhibited by high and low luminal Na^+ concentrations, respectively. The Na^+/K^+ ATPase maintain a high extracellular Na^+ concentration in intestinal cells, ensuring that Na^+ moves down its concentration gradient into the cell. Glucose follows Na^+ into enterocytes through SGLT-1 and leaves the cell at the basal surface through GLUT 2 to enter the interstitium, and thus, the capillaries. Because energy is expended to actively transport Na^+ outside of the cell by the Na^+/K^+ ATPase, establishing the gradient that drives the transport of glucose, intestinal glucose transport is an example of secondary active transport.

Fructose transport relies on a different, Na^+ independent, mechanism (Figure 1-7). It enters the enterocytes from the intestinal lumen through facilitated diffusion provided by GLUT5. Once inside the mucosal cells, some fructose is converted to glucose, and like glucose, the remainder enters the interstitium by GLUT 2.

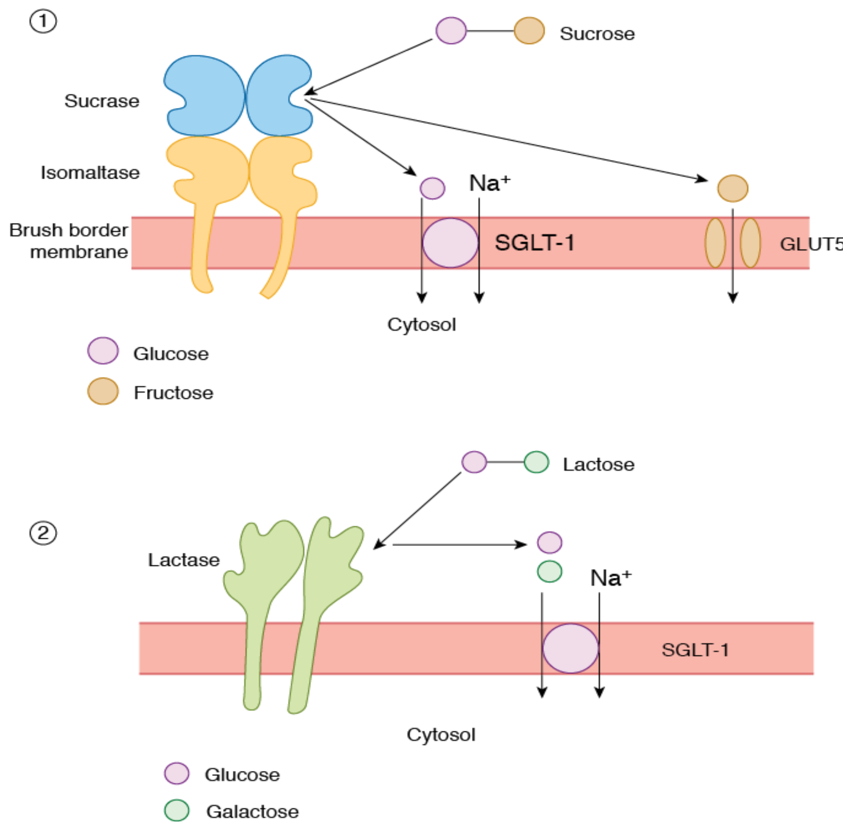

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Figure 1-7. Brush border digestion and assimilation of the disaccharides sucrose (panel 1) and lactose (panel 2). SGLT-1, sodium-glucose cotransporter-1. (5)

Proteins and Nucleic Acids

The digestion of proteins begins in the stomach. Inactive precursors (proenzymes) called pepsinogens are activated by gastric acid, yielding the active enzyme pepsin. Bonds between aromatic amino acids and a second amino acid are hydrolyzed by pepsin; polypeptides of wide-ranging sizes are produced. Pepsins are inactivated, however, when the gastric contents mix with the alkaline pancreatic secretions in the duodenum and jejunum. Pepsins have an optimum pH of 1.6 to 3.2.

Strong proteolytic enzymes of the pancreas and intestinal mucosa continue to digest polypeptides in the small bowel following peptic activity in the stomach. Endopeptidases, including the chymotrypsins, trypsin, and elastase, target inferior peptide bonds in the peptide molecule. Active endopeptidases are initially secreted as

inactive precursors, and only become active once they reach their site of action in the duodenum (Figure 1-8). The brush boarder hydrolase, enterokinase, converts trypsinogen to its active form trypsin once the pancreatic juice reaches the duodenum. Formation of trypsin has an auto-catalytic quality, meaning that trypsin can itself activate trypsinogen. Trypsin activates chymotrypsinogens into chymotrypsin, as well as other proenzymes into their active forms.

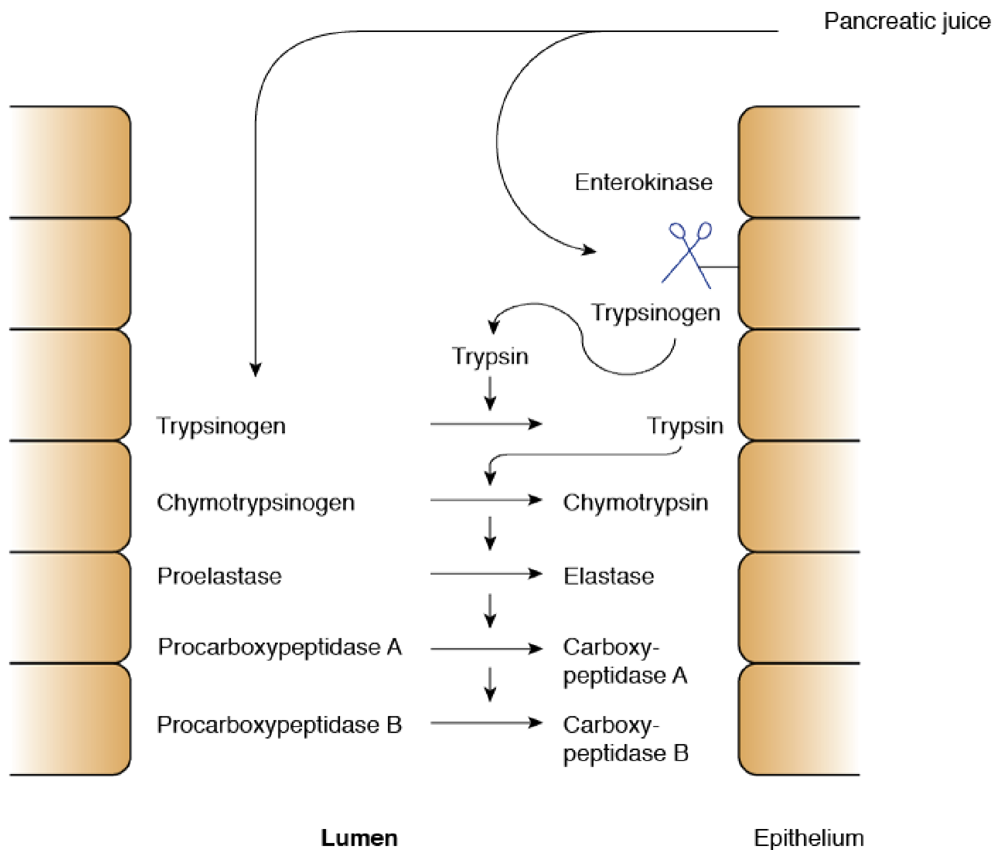


Figure 1-8. Mechanism to avoid activation of pancreatic proteases until they are in the duodenal lumen. (5)

Exopeptidases are carboxypeptidases produced by the pancreas. These enzymes cleave amino acids at the carboxyl ends of the polypeptides. While some free amino acids are released in the intestinal lumen, others are liberated at the brush boarder of

mucosal cells by carboxypeptidases, aminopeptidases, endopeptidases, and dipeptidases. Enterocytes actively transport some di- and tripeptides into the cytoplasm where intracellular peptidases carry out hydrolysis. Therefore, the intestinal lumen, the brush boarder, and the cytoplasm of mucosal cells each act as the final site for polypeptide digestion to free amino acids.

Amino acids enter enterocytes through at least seven different transport systems. Similar to the cotransport of Na^+ and glucose, amino acids are cotransported with Na^+ into enterocytes by five of the amino acid transporters. Chloride is essential for two of those five.

Two of the seven transport systems are Na^+ independent. Instead of requiring Na^+, the PepT1 system utilizes H^+ to transport di- and tripeptides into intestinal cells (Figure 1-9). At the apical membrane, the sodium/hydrogen exchanger supplies the H^+ for cotransport by PepT1. Free amino acids liberated by intracellular peptidases, and those absorbed from the intestinal lumen and brush boarder, exit the basolateral surface of the cell by at least five transport systems, entering the hepatic portal blood.

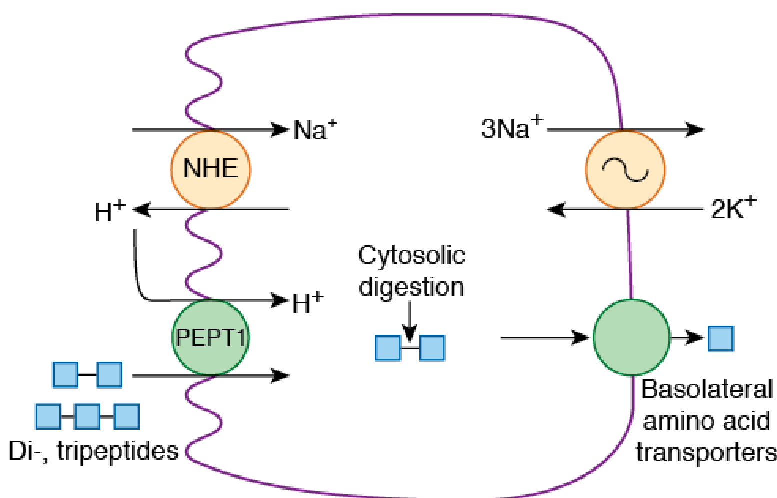


Figure 9. Disposition of short peptides in intestinal epithelial cells. (5)

While the absorption of amino acids occurs quickly in the duodenum and jejunum, it is slow in the ileum. About 2-5% of the amino acids evade intestinal digestion and absorption, with colonic bacteria eventually digesting some of it. Of the protein in the feces, virtually none is of a dietary source, coming rather from bacteria and cellular debris.

Pancreatic nucleases cleave nucleic acids into nucleotides. Enzymes thought to be positioned on the luminal surfaces of mucosal cells split nucleotides into nucleosides and phosphoric acid. The nucleosides undergo further cleavage into their appropriate sugars and purine and pyrimidine bases. The bases are absorbed by active transport.

Lipids

Digestion of fats mostly begins in the duodenum. Pancreatic lipase acts on emulsified fats, easily hydrolyzing the 1- and 3-bonds of the triglycerides. However, this lipase acts slowly at the 2-bonds, generating free fatty acids and 2-monoglycerides as its main products. An amphipathic helix covers the active site of pancreatic lipase, and its activity is enhanced when the helix is bent back. A component of the pancreatic secretion, colipase facilitates opening of the helix cover by binding to the –COOH-terminal domain of the pancreatic lipase. Trypsin activates colipase in the intestinal lumen from its proenzyme form.

Cholesterol esterase is a bile salt-activated lipase secreted by the pancreas. Although 10-60 times less active than pancreatic lipase, it has more varied activity, hydrolyzing cholesterol esters, esters of fat-soluble vitamins, phospholipids, and triglycerides.

Bile salts, lecithin, and monoglycerides emulsify fats in the small intestine, enhancing the ability of these quite insoluble molecules to reach the mucosal cell surface. Micelles form through the interaction of lipids and bile salts following contraction of the gallbladder, which creates a high concentration of bile salts in the intestine (Figure 1-10). Micelles are cylindrical species that sequester and help solublize lipids, and provide a vehicle for lipid transport to the enterocytes. They have a hydrophobic centre typically composed of fatty acids (FA), monoglycerides, and cholesterol. The fatty acids diffuse out of the micelles, coming in contact with the mucosal brush boarder.

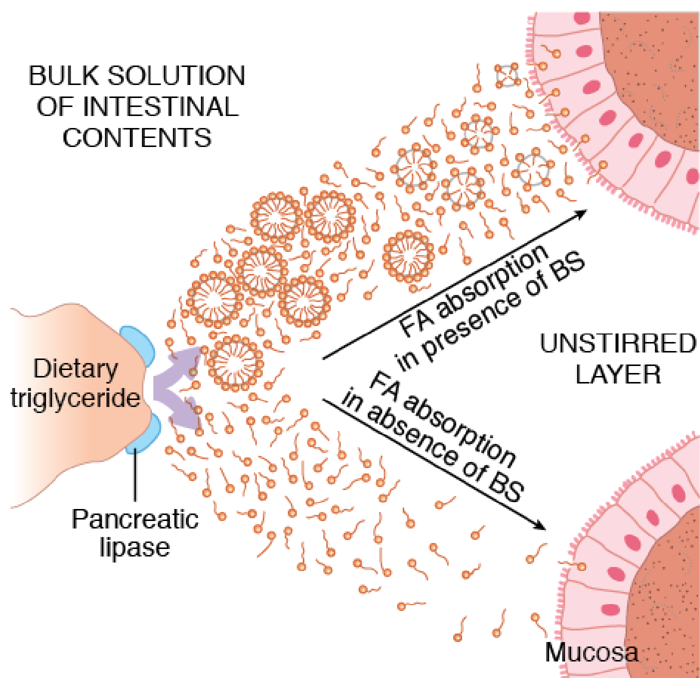
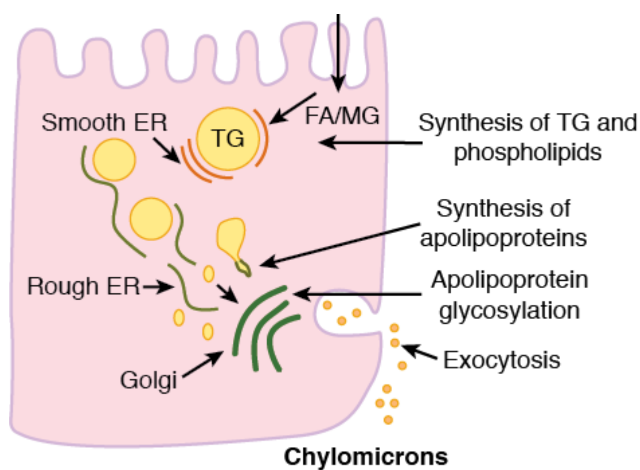


Figure 1-10. Lipid digestion and passage to intestinal mucosa. (5)

Opposing the traditional view that lipids passively diffuse into intestinal cells, more recent evidence suggests that carriers are involved. The concentration gradient favouring lipid entry into enterocytes is maintained by quick esterification of lipids inside the cells; although, not all fatty acids are esterified. Relatively water-soluble fatty acids,

those with less than 10 to 12 carbon atoms, leave the enterocyte unmodified and are actively transported into the portal blood. However, being too insoluble, fatty acids with greater than 10 to 12 carbons are re-esterified to triglycerides within enterocytes. Chylomicrons are then formed by covering the triglycerides (TG) and cholesterol esters with phospholipid, cholesterol, and protein. The chylomicrons leave the cell and enter the lymphatic system (Figure 1-11).



Source: Barrett KE, Barman SM, Boitano S, Brooks HL: *Ganong's Review of Medical Physiology:* www.accessmedicine.com
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Figure 1-11. Intracellular handling of the products of lipid digestion. (5)

Mostly occurring in the smooth endoplasmic reticulum, absorbed 2-monoglycerides undergo acylation in mucosal cells to form most of the triglyceride in these cells. Glycerophosphate also contributes to triglyceride formation, and is a player in chylomicron formation after conversion into glycerophospholipids. The rough endoplasmic reticulum houses glycerophosphate acylation and the formation of lipoproteins. The final addition to the chylomicrons takes place in the Golgi apparatus, where carbohydrates are added to the proteins. Chylomicrons exit the basal or lateral aspect of the cell through exocytosis.

Humans absorb 95% or more of their dietary fat on a moderate fat intake. The duodenum and jejunum absorbs the majority of long-chain fatty acids, with the ileum absorbing lesser amounts.

Fluid and Electrolytes (6)

The greatest amount of water absorption occurs at the villous tips in mature epithelial cells of the small intestine. Immature cells found at the villous crypts are responsible for the majority of water secretion. Movement of water either into or out of the intestinal lumen is a passive process, both secondary and proportional to ion and nutrient transport. Intestinal contents are kept iso-osmotic with plasma under normal conditions. Water and ions are transported through transcellular and paracellular routes. The former pathway is the major one and involves specialized water channels called aquaporins. Paracellular transport from the lumen to the mucosa occurs through leaky epithelial tight junctions that become less leaky at the ileum.

In the jejunum, transcellular Na^+ absorption is carried out by cotransport with nutrients, or by Na^+-K^+ exchange. A paracellular pathway exists for Na^+ and Cl^- absorption. The proximal duodenum secretes HCO_3^- ions; however, the jejunum absorbs a large quantity of HCO_3^- and Cl^- ions. While absorbing Cl^-, the ileum secretes HCO_3^-. For each molecule of glucose that SGLT1 takes up, two Na^+ ions are also transported. Passive paracellular transport is the main mechanism of K^+ absorption from the lumen of the small intestine.

Hormones, such as angiotensin II and aldosterone, and neurotransmitters control water and electrolyte absorption. During states of dehydration, angiotensin II and aldosterone are released, promoting NaCl absorption in the intestine.

Peptide Hormones of the Small Intestine (7)

Gastrin

Gastrin is the major hormone responsible for gastric acid secretion in the stomach. It has also been found to have some growth-promoting activities as well. Although gastrin is mostly produced in endocrine cells of the gastric antrum, smaller amounts are produced in other regions of the GI tract, including the entire length of the small intestine. A precursor called preprogastrin is synthesized prior to the active hormone. Multiple, active forms of gastrin are generated through enzymatic processing of preprogastrin. Specialized endocrine cells called G cells secrete gastrin into the bloodstream after a meal, a process heavily influenced by stomach pH. Both gastrin and Cholecystokinin (CCK) have related receptors, constituting the gastrin-CCK receptor family. The CCK-1 receptor has a 1000-fold higher affinity for CCK than for gastrin, and is located in the gallbladder and pancreas.

Cholecystokinin

CCK has a multitude of actions, helping to coordinate the ingestion, digestion, and absorption of dietary nutrients. Binding to CCK-1 receptor on the gallbladder, pancreas, smooth muscle of the stomach, and peripheral nerves, CCK stimulates gallbladder contraction and pancreatic secretion, regulates gastric emptying and bowel motility, and induces satiety. I cells of the small bowel produce CCK, which is released into the circulation after ingestion of a meal. The major components of food that stimulate CCK secretion are lipids and protein. As with gastrin, active CCK forms are produced through post-translational processing of a preprohormone.

Secretin

Enteroendocrine cells of the small intestine called S cells generate secretin, a hormone structurally similar to glucagon and Vasoactive Intestinal Polypeptide (VIP), and that acts through G protein-coupled receptors. The duodenum releases secretin when acid is present, resulting in secretion of pancreatic fluid and bicarbonate. In the duodenum, bicarbonate neutralizes gastric acid and raises the duodenal pH. A negative feedback system responds to the elevated pH and secretin release ceases. Through its enterogastrone activity, secretin is also secreted when fat is present in the intestinal lumen to inhibit gastric acid release; gastrin release and gastric motility are inhibited as well.

Vasoactive Intestinal Polypeptide

VIP is a powerful vasodilator, increasing blood flow to the gut and causing smooth muscle relaxation and epithelial cell secretion. This neuromodulator acts at a local level through G protein-coupled VIP receptors after being released from nerve terminals. VIP production largely occurs in peripheral-enteric and central nervous system neurons. Being widely distributed, VIP has effects on many different organ systems. In intestine, it stimulates epithelial fluid and electrolyte secretion. Other GI-related VIP actions include relaxation of GI smooth muscle, a result of inhibited rhythmic depolarizations of the smooth muscle membrane potential and subsequent relaxation.

Glucagon

There are intestinal L cells in the ileum and colon, as well as pancreatic alpha cells, that produce and secrete glucagons. Preproglucagon and glucagon-like peptides (GLPs) are encoded by the glucagon gene. Prohormone convertases carry out tissue-specific peptide processing in the pancreas and intestine, generating glucagon and GLP-1 and GLP-2, respectively. Both glucagon and GLP-1 regulate glucose homeostasis.

Glucose- Dependent Insulinotropic Polypeptide

Following ingestion of glucose or fat, GIP is released into the blood from mucosal K cells of the small intestine. Under conditions of hyperglycemia, and not otherwise, GIP binds to its pancreatic beta cell receptor, leading to increased intracellular calcium concentrations and resultant insulin secretion. Adipocytes also possess GIP receptors, through which GIP increases triglyceride storage.

Pancreatic Polypeptide Family

The pancreatic polypeptide (PP) family of peptides includes PP, NPY, and peptide tyrosine tyrosine (PYY). Specialized endocrine cells in the pancreas (PP cells) store and release PP. In contrast, NPY is a neurotransmitter found throughout the central and peripheral nervous systems. Enteroendocrine cells throughout the GI tract contain PYY, but the ileum and colon have the highest concentrations of PYY. The PP family has endocrine, paracrine, and neurocrine regulatory actions. PP inhibits pancreatic exocrine secretion, gallbladder contraction, and gut motility. PYY inhibits vagally stimulated gastric acid secretion and other motor and sensory functions, as well

as food intake. Increased food intake is one of the centrally mediated effects of NPY. In the periphery, NPY modulates vascular and GI smooth muscle function.

Somatostatin

Somatostatin has been found in virtually every organ of the body. In the GI system, D cells in the gastric and intestinal mucosa, islets of the pancreas, and enteric neurons produce somatostatin. The effects of somatostatin are mostly inhibitory. Decreased cAMP, Ca^{2+} inhibition, or K^{+} channel opening mediate somatostatin's inhibitory actions. The broad GI effects of somatostatin include regulation of gastric acid secretion, reduced pepsinogen release, inhibition of pancreatic enzyme, fluid, and bicarbonate secretion, reduced bile flow, and regulation of GI motility. Reduced splanchnic blood flow, decreased intestinal nutrient and fluid transport, and inhibited tissue growth and proliferation are also effects of somatostatin.

Motilin

Motilin is produced by endocrine cells of the duodenal epithelium. The release of this peptide is synchronized with the migrating motor complex (MMC) under fasting conditions. Phase III motor contractions produced in the antroduodenal area are regulated by heightened motilin concentrations in the blood. The esophagus, stomach, and small and large intestines have motilin receptors on their smooth muscle cells. Binding of motilin to its receptor results in propulsive activity.

Small Bowel Transplantation

Intestinal transplantation is currently indicated for patients with irreversible intestinal failure and who have complications with parenteral nutrition, cannot adapt to restrictions in quality-of-life that intestinal failure imposes, or have a high probability of death without excision of the native intestine (8). The most common diagnosis prior to transplantation is the short bowel syndrome (SBS), and sepsis remains the most common cause of death following transplantation (9). Both the number of procedures performed and favourable outcomes have increased within recent years.

Early efforts to successfully transplant the small intestine proved difficult within both the experimental and clinical settings. Technical complications, host rejection, and graft-versus-host disease (GVHD) were the most significant hurdles to successful engraftment (10). As surgical expertise improved, more potent immunosuppressive regimens became available, and prophylaxis for infections and GVHD was made standard, however, results improved. For transplants performed worldwide between February 1995 and February 1997, 1-year graft/patient survival for intestinal grafts was 55%/69% (11). These statistics improved to 65%/77% for the period of 1998 to 2003 (9). In the United States, 1-year graft/patient survival for intestinal grafts occurring from 2006 to 2007 was 78.9%/89.3%, a statistic indicating that small bowel transplantation is approaching the success of other transplanted intra-abdominal organs over the short-term (12). Unfortunately however, long term survival remains low. Five-year graft/patient survival rates were just 39.6%/57.9% between 2002 and 2007 in the United States (12). And while 10-year patient and graft survival rates are regularly more than 50% for the kidney, liver, and heart, 10-year patient survival for isolated intestinal transplants is only 46% (12).

Likely a result of increased short-term success, the number of transplants per year has been increasing over the last 20 years. Globally, only 11 cases were reported in 1990, while 140 procedures were performed in 2003 (9). OPTN/SRTR reported a 21.1% increase in the number of intestinal transplants from 2007 to 2008 (12).

Intestinal Failure and Short Bowel Syndrome

"Intestinal failure results from obstruction, dysmotility, surgical resection, congenital defect, or disease-associated loss of absorption and is characterized by the inability to maintain protein-energy, fluid, electrolyte, or micronutrient balance (13)." Along with being comprehensive and precise, this definition also recognizes that intestinal length does not solely determine its function; a shorter length of healthy bowel may function better than a longer length of diseased bowel (13). Moreover, outcomes in clinical practice depend on a variety of prognostic factors, including the quality of intestine remaining and if the large intestine has been left intact (13).

There are three main categories used to group the causes of intestinal failure according to pathogenesis: gut resection with short bowel syndrome (SBS), GI-associated neuromuscular disease, and congenital diseases involving the bowel epithelium (14). Within the context of intestinal failure, the underling etiology differs between children and adults (15). Short bowel syndrome is a major cause among both demographics, however (15). One definition for SBS, created by a panel of experts, places it under the broader category of intestinal failure: "short-bowel syndrome-intestinal failure results from surgical resection, congenital defect or disease-associated loss of absorption and is characterized by the inability to maintain protein-energy, fluid,

electrolyte or micronutrient balances when on a conventionally accepted, normal diet (13)."

Following extensive resection of the small bowel, the remaining small bowel undergoes a physiological adaptation process (14). The bowel diameter and wall thickness increases as the muscularis hypertrophies, and mucosal hyperplasia also ensues. During mucosal hyperplasia, enterocyte numbers are up-regulated per unit length of intestine, and the rate of enterocyte proliferation, villus height, and crypt death is increased. Expanded mucosal mass results from epithelial hyperplasia. A complex array of hormones and luminal factors regulate bowel mucosal growth.

Both medical and surgical strategies are used to manage intestinal failure (15). In the majority of cases, parenteral nutrition (PN) is still the crux of treatment. However, additional approaches, such as new therapeutic agents (growth factors) and surgical techniques focused on maximizing intestinal length and function are under development. Although not a novel concept, small bowel transplantation has become an increasingly feasible therapeutic option for patients who cannot tolerate the complications of PN.

Parenteral Nutrition and its Complications

As stated previously, PN is the routine therapy for patients with irreversible intestinal failure (15). Sustaining life by supplying the body with essential nutrients and fluid, PN supports physical and cognitive growth. The adaptive response of the intestine and repair mechanisms are also facilitated by the energy and nutrients provided by PN. Hospital patients whose GI tract cannot adequately absorb life-sustaining fluids and nutrients, but who are stable and ready for discharge, are candidates for home

parenteral nutrition (HPN) (16). In those individuals requiring life-long PN, a crucial balance between metabolic needs and therapy-associated complications exists (15).

There are a host of complications related to PN, some being more serious than others (16). Tunnel and exit site infections are common, and sepsis can develop from the catheter and other sources. Catheter dislodgment, air embolism, withdrawal occlusion, and pump malfunction represent the mechanical complications. There is also a risk of metabolic bone disease for patients receiving long-term PN. Another long-term complication, as well as the most worrisome, is liver failure.

Liver and biliary system disorders are life-threatening complications accompanying PN (17). Of the children requiring long-term TPN for intestinal failure, 40-60% develop liver disease. Liver disease manifests in 15-40% of adults on HPN (18). The term “PN-associated liver disease” (PNALD) recognizes the multifactorial nature of liver dysfunction in patients receiving PN (17).

There are 3 types of hepatobiliary disorders related to PN: steatosis, cholestasis, and gallbladder sludge/stones (17). Some disorders are more common in either adult or pediatric populations. Accumulation of liver fat (steatosis) is largely restricted to adults, and is typically not serious. Usually serum aminotransferase, serum alkaline phosphatase, and bilirubin concentrations elevate within 2 weeks of PN initiation, but may normalize with continued PN. Although most patients are asymptomatic, hepatic steatosis can progress to fibrosis or cirrhosis in patients requiring long-term PN.

Cholestasis occurs most often in children, but also in adults on long-term PN (17). This condition results as a consequence of aberrant secretion of bile or blocked biliary flow. Serum conjugated bilirubin levels >2 mg/dL indicate cholestasis, which is a serious complication of PN, as it may progress to cirrhosis and liver failure.

Gallstones or gallbladder sludge results from gallbladder stasis during PN and may lead to cholecystits (17). Not necessarily a direct result of PN, but rather diminished enteral feeding, both adults and children can develop gallbladder sludge/stones.

The etiology of PNALD is multifactorial and can differ between pediatric and adult patients (18). Prematurity, low birth weight, PN duration, SBS requiring multiple laparotomies, recurrent sepsis, and suspended enteral feeding are important factors in infants who develop PNALD. Significant contributors in adults are age, length of time on PN, total caloric intake, and lipid or glucose overload. Both children and adults can experience hepatic steatosis and cholestasis, and these conditions are related to lipid emulsions, choline deficiency, and manganese toxicity.

Indications for Small Bowel Transplantation

Patients with irreversible intestinal failure are offered small bowel transplantation (SBTx) according to their success or failure of their adaptation to PN (8). Formal indications for intestinal transplantation are broken down into two classifications: medically-approved indications, and non-traditional indications (19). The former indications include loss of major routes of venous access, repeated occurrence of catheter-associated life-threatening sepsis, fluid and electrolyte abnormalities despite maximal medical therapy, and PN-associated cholestatic liver disease. The latter category includes diffuse mesenteric thrombosis, benign/low-grade malignant tumors involving the mesenteric root, and abdominal catastrophes.

Currently, owing to the vaguely defined Medicare criteria, there exists some controversy about when a person should be offered transplantation (20). As a result, many patients receive grafts late, negatively influencing outcomes. However, a strategy

allowing for earlier referral in patients considered high risk for complications of PN is under development (20).

What Disease States Require Transplantation?

The majority of adults who undergo intestinal transplantation have SBS (8). Crohn's disease, mesenteric vascular accidents, trauma, volvulus, or surgical complications are the leading causes. In some cases of locally advanced mesenteric tumors, the sole treatment option is to exenterate and transplant the intestine (8). Most of the children requiring transplantation also have SBS (8). Surgical resection is usually the cause of SBS. The leading diagnosis' in the pediatric population are necrotizing enterocolitis (26%), gastroschisis (16%), atresia (10%), and volvulus (9%) (21). A lower percentage of children have congenital enterocyte disorders, causing motility abnormalities, infantile diarrhea, or malabsorption from polyposis.

Types of Grafts

Any small bowel transplantation involves the jejunoileal segment of the small intestine by definition (8). An isolated intestinal transplant refers to transplantation of the jejunoileum on its own, without co-transplantation of other abdominal organs. With that said, co-transplantation of other organs with the intestine is not infrequent.

End-stage liver disease necessitates liver replacement as well (8). This is achieved in two ways: either as a composite allograft, or by separate implantation of the intestine and liver from the same donor. Primarily reserved for small children, the pancreas and duodenum are commonly transplanted with the liver and intestine to accommodate en bloc engraftment and to obviate biliary reconstruction.

In patients with coexisting intestinal disorders or diseased other organs, replacement of the entire GI tract is required (8). Multivisceral (composite visceral or multiorgan) transplants often refer to transplants in which the stomach accompanies the small bowel.

Patients are given enteral nutrition soon after the operation through a feeding tube and slowly transition to oral feeding (8). Monitoring of the intestinal mucosa is achieved through tissue biopsy at the ileostomy site constructed during the operation (8). Reversal of the ileostomy in patients that have preserved colon function, or whose allograft included one, occurs once graft function stabilizes.

Immunosuppression

Immunosuppression has advanced in recent years, resulting in improved outcomes following intestinal and multivisceral transplantation in recent years (22), resulting in increased survival and quicker restoration of nutritional autonomy and quality of life. Despite each transplant centre having its own immunosuppressive protocol, tarcolimus and corticosteroids are generally considered the universal regimen. Unfortunately, both drugs are associated with serious complications when used over the long term (23). Tarcolimus is provided immediately after the surgery for maintenance immunossupression by enteric delivery (23). Other drugs added to the maintenance therapy are mycophenolate mofetil and rapamycin (22). When added to the maintenance phase, rapamycin significantly improves graft survival, and significantly decreases the incidence of early graft rejection and graft loss by fulminant rejection (24). Another relatively recent advancement has been the use of induction immunosuppressive agents to reduce the risk of early and late graft rejection (22). The

induction agents alemtuzumab, basiliximab, and daclizumab (monoclonal antibodies), and thymoglobulin (polyclonal antibody) are administered prior to, or during, the surgery (23).

Complications

Surgical complications have become rarer over the last decade as surgical experience has increased (8). The rates of technical failure, graft thrombosis and ischemia have been decreasing; medical and immunologic causes of graft non-function are now more common than surgical complications.

Graft rejection is one of the leading causes of graft failure and patient death following SBTx (9). Many factors have contributed o the reduction in the rates of rejection, and prompt detection has proved critical for reversal of the rejection process (8). Although invasive, endoscopic surveillance biopsy remains the only definitive diagnostic tool for rejection (8). The use of newer medications, such as monoclonal interleukin-2 antagonists, polyclonal antithymocyte antibodies, and sirolimus has decreased the rate and severity of early acute rejection at multiple transplant centers (8). Once occurring at a rate of 70-90%, acute cellular rejection in the first 90 days now only affects 33-50% of recipients (8).

Infections continue to persist as a common complication after transplantation (8). As the presentation of diarrhea may indicate either rejection or viral and bacterial enteritis, it is important to distinguish these conditions by biopsy and evaluation by a knowledgeable pathologist. The manifestations of adenovirus, calicivirus, *Clostridium difficile*, and cytomegalovirus infections may all resemble rejection. Pediatric transplant

patients initially had a high incidence of lymphoproliferative disorder, owing to Epstein-Barr virus, but this is no longer much concern.

Static Organ Preservation and Ischemia/Reperfusion Injury

The success of organ transplantation is intimately linked to our ability to preserve the organ. Organ harvest, tissue matching, organ transport, and transplant team preparation all contribute to an unavoidable time period between donor death and recipient transplantation. Organ preservation maintains graft viability over this time.

During donor organ retrieval, unfractionated heparin is administered intravenously right before aortic cross clamping (25). The harvest team then places cold water or crushed ice into the body cavities after the heart has stopped beating. Following this step, an intravascular flush with hypothermic preservation solution commences, rapidly cooling the organs to below 10°C. The temperature and viscosity of the perfusate affects the rate and efficiency of the vascular wash-out.

Collins developed the first successful preservation solution used for static cold storage (25). The creation of Eurocollins (EC) solution followed after a number of modifications to Collin's original formulation, and became the standard clinical cold storage solution for the next 20 years. The University of Wisconsin (UW) solution, developed by Belzer, eventually replaced EC and became the new 'gold standard' organ preservation solution. Continued optimization of static organ preservation is essential, as the ability of an organ to tolerate hypothermic storage depends on the composition of the preservation solution (26). Regardless of which preservation solution is used, however, the goal remains the same: to prevent ischemia/reperfusion injury (25).

Mechanism of Ischemic Damage

A number of pathological biochemical processes occur in response to tissue ischemia. These events disrupt cellular and membrane function, leading to intracellular fluid retention and eventual cell death (27). Under normal circumstances, mammalian tissues have an adequate supply of oxygen and ATP is efficiently generated through aerobic respiration. However, in the event of reduced or complete cessation of blood flow, insufficient amounts of oxygen reaches the tissues and hypoxia ensues. Cellular respiration switches from aerobic to anaerobic under hypoxic conditions, resulting in a reduced efficiency and ability to produce ATP (28). A supply and demand issue arises as the ability to generate ATP cannot meet the need of energy-consuming cellular functions. In the face of rapidly declining energy stores, the Na+/K^+ATPase fails to maintain transmembrane ion gradients (29). The resulting cell membrane depolarization is a consequence of unopposed sodium ion influx and potassium ion efflux into and out of the cell, respectively. Voltage-dependent Ca^{2+} channels respond to the altered membrane voltage by allowing a massive influx of calcium ions into the cell, leading to cell membrane and organelle phospholipid hydrolysis by calcium-activated phospholipases (30). Together, Na+/K^+ATPase dysfunction and impaired cell membrane integrity allow for increased influx of sodium into the cells; water parallels sodium movement, causing cellular edema and eventual lysis and death (28).

The Effects of Tissue Ischemia

Lesions of the mucosa form when tissues are inadequately oxygenated (31, 32). One research group detailed the sequence of morphological changes associated with normothermic intestinal ischemia in a dog model (32). Subepithelia, fluid-filled

Gruenhagen spaces formed at villous apices within 0.5 to 1 hour after partial superior mesenteric artery (SMA) occlusion, causing epithelia lifting from the basement membrane. The development of subepithelial spaces progressed over longer ischemic periods, extending down toward the basal villous surface. Complete SMA blockage for 1 hour or partial occlusion for more than 4 hours caused severe damage; villi were denuded of epithelium, and the structural integrity of the lamina propria was compromised. Park *et al* classified the sequence of morphologic intestinal deterioration in rats undergoing increased normothermic ischemic periods (33). As the ischemic time progressed, increased levels of injury were seen. Epithelial lifting at the villus apex and extension of the lifting down the lateral aspects of the villus occurred early, but only after 20 minutes of ischemia. Complete denudation, cellular loss in the villus interior, and crypt infarction followed. Necrosis of the transmural and transmucosal tissue layers presented last, and up to 90 minutes of total artery occlusion. The onset and rate of progression of ischemic damage does not appear uniform throughout the entire small intestine, however (34).

In the arena of small bowel transplantation, a number of researchers studied the effects of hypothermic preservation of the intestine. Significant reductions in the rate of cellular metabolism are achieved during hypothermia. Tissue histology following 10 to 24 hours of hypothermic storage revealed mucosal damage on par with that observed after just 60 to 90 minutes of warm ischemia (35-39). Similar findings were seen regarding the mild morphologic damage incurred when intestines were cold-stored for less than 9 hours (35-37, 40).

Reperfusion Injury

It is well established that reestablishment of blood flow to previously ischemic intestinal tissue can exacerbate the intestinal damage previously obtained. Within just 10 minutes after reperfusion, histologic damage increases (41). In a foundational

study using cat small bowel, Parks and Granger found that compared to 4 hours of ischemia alone, intestinal damage was significantly worse in the group subjected to 3 hours of ischemia followed by 1 hour of reperfusion (42). Supporting these results is the documentation that three hours of ischemia in dogs resulted in mild to moderate mucosal injury, but reperfusion for 1 hour produced moderate to severe damage (43).

The Role of Oxygen Free Radicals

Mitochondria normally reduce oxygen to water by way of the cytochrome system through a tetravalent redox reaction (44). Another pathway also exists, but accounts for only 1 to 5% of reduced oxygen. In the lesser pathway, xanthine oxidase (XO) catalyzes the univalent reduction of molecular oxygen, forming the oxygen free radical (OFR), superoxide ($\cdot O_2$) (44-47). Oxygen free radicals (OFRs) are the product of partial reduction of oxygen. They contain one or more unpaired electrons, making them highly unstable and readily reactive (27, 44).

Once superoxide is formed, a sequence of subsequent reactions commences, yielding more powerful reactive oxygen species. In fact, superoxide has minimal cytotoxic activities by itself. Through a dismutation reaction catalyzed by the enzyme superoxide dismutase (SOD), superoxide undergoes conversion to hydrogen peroxide (H_2O_2), a molecule also directly produced from O_2 through divalent reduction. In the

iron-catalyzed Haber-Weiss reaction, H_2O_2 and $\cdot O_2$ react, forming the hydroxyl radical ($\cdot OH$) (44, 46). The small bowel contains ample amounts of iron in the form of ferritin, which contains a Fe^{3+} form of iron that reacts with superoxide. Once freed, Fe^{2+} then reacts with hydrogen peroxide to yield the hydroxyl radical (48).

The hydroxyl radical has potent cytotoxic effects (44, 46). Through oxidation of sulfhydryl compounds, hydroxyl radical production results in enzyme dysfunction and inactivation of membrane transport proteins. Damage also occurs through reaction with DNA, and cytochromes and other proteins. Cell membranes contain polyunsaturated fatty acids that are attacked by $\cdot OH$, leading to lipid peroxidation. Formation of membrane lipid peroxides result, destabilizing the lipid membrane, leading to potential cell death (46).

In addition to being converted to $\cdot OH$, hydrogen peroxide also reacts to form another cytotoxic OFR. In a reaction catalyzed by the enzyme myeloperoxidase (MPO), the chloride anion reacts with H_2O_2, generating hypochlorous acid (HOCl) and water (46, 49). *N*-chloro-derivatives are produced through the chlorinating and oxidating properties of hypochlorous acid. The cytotoxic effects of HOCl are mediated by *N*-chloro-derivatives, which damage DNA by altering purine bases, and alter amino acid structure, crippling protein function.

Tissues have endogenous OFR scavenger systems that limit the levels of cytotoxic OFRs in the body. Two such scavengers, superoxide dismutase and catalase, act in tandem to prevent $\cdot OH$ formation (44). After SOD facilitates the dismutation of $\cdot O_2$ to H_2O_2, catalase reduces H_2O_2 to water. Although endogenous OFR scavengers manage to control physiological OFR generation, they become saturated and fail to protect tissues in the setting of pathological increases in OFR formation. Such an event occurs

when previously ischemic tissues are reperfused. Indeed, OFR generation increased hundreds of percentage points following reperfusion after an initial cold storage period in rat small intestine, compared to non-ischemic controls (40).

Villi of the intestinal mucosa have a very high concentration of XO (50), the enzyme primarily responsible for OFR production during ischemia-reperfusion (49, 51, 52). Acting in the oxidation of purines, xanthine dehydrogenase (XD) is the main form of XO in normal cells (49); however, XD undergoes conversion to XO when tissues become ischemic (53, 54). Ischemic intestinal tissue has copious amounts of hypoxanthine (HX), which XO oxidates to produce $\cdot O_2^-$ and H_2O_2 (49). Large quantities of hypoxanthine are generated during ischemia, as ATP is quickly catabolized to AMP, which is further broken down to adenosine and then inosine. Further degradation of inosine produces hypoxanthine (27, 49, 51, 52). Supporting the role of XO and HX in ischemia-reperfusion (IR) injury, dog intestines exhibited similar intestinal injury to IR injury when non-ischemic tissues were intraluminally infused with XO and HX (55). Moreover, the XO inhibitor, allopurinol, reduced the injury. Others have showed that administration of XO inhibitors lessens intestinal damage in the setting of IR (56, 57).

With accumulation of XO and HX during ischemia, the intestinal mucosa is ready to produce OFRs upon the reintroduction of blood and thus oxygen (52). Without oxygen, the XO-mediated generation of the OFRS, $\cdot O_2^-$ and H_2O_2, would not take place. As discussed earlier, $\cdot O_2^-$ undergoes dismutation to form H_2O_2, which then converts to the potent $\cdot OH$ through the Haber-Weiss reaction. Many studies have implicated ORFs in the deleterious effects seen in IR injury (51, 57).

The Role of Neutrophils

Oxygen free radicals do not solely inflict direct cytotoxic damage and dysfunction during ischemia-reperfusion; they are also responsible for the activation and infiltration of neutrophils early in reperfusion (46, 49). Neutrophils themselves are cytotoxic and tissue-damaging in the realm of IR, as they infiltrate previously ischemic tissues on a local level during reperfusion. Expression of myeloperoxidase (MPO) is near completely restricted to neutrophils that convert H_2O_2 to HOCl (46); therefore, MPO acts as a biological marker for activated neutrophils. Using MPO as a measure of neutrophil infiltration, one study documented increased infiltration following reperfusion of ischemic tissue (58). The same study implicated XO- produced OFRs as playing a role in neutrophil infiltration after reperfusion; SOD or allopurinol pretreatment lessened neutrophil invasion. Cicalese *et al* demonstrated significant increases in activated neutrophils, measured by direct MPO staining, after reperfusion of small intestine preserved using cold Ringer's lactate solution for 2 hours (40). A strong correlation was found between neutrophil influx and amount of OFRs produced, and the grade of intestinal injury. Research has also showed that neutrophils have direct interactions with the endothelium during IR (35, 59-61).

Once activated, neutrophils inflict damage through multiple mechanisms. They restrict blood flow in mucosal capillaries and venules, described as the "no reflow" phenomenon (explained in a later section). Neutrophils also breakdown the endothelial basement membrane and extracellular matrix by releasing proteolytic enzymes, such as proteases, elastase, and collagenases (49). Another consequence of neutrophil activation is further production and release of OFRs. With just one gram of intestinal tissue containing about 10 million neutrophils, the intestine has a huge capacity for OFR

production that far exceeds XO-mediated generation alone (58). NADPH oxidase is the enzyme responsible for $\cdot O_2^-$ production within activated neutrophils (46, 49). Once generated, $\cdot O_2^-$ takes part in additional reactions (outlined above), yielding the more potent and cytotoxic OFRs ·OH and *N*-chloramines (49).

The contribution of neutrophils to the functional and structural damage seen in IR injury has been largely elucidated by studies employing neutropenic reperfusion. Following 1 hour of incomplete ischemia and 2 hours of reperfusion, Hernandez *et al* showed that neutropenia induction in cats by pre-treatment with anti-neutrophil serum (ANS) significantly reduced intestinal permeability (59). Another research group documented elevated tissue ATP levels in neutropenic rats after 30 minutes of intestinal ischemia and reperfusion, compared to non-neutropenic controls (62). Brown *et al* rendered rat intestines ischemic for 90 minutes *in vivo* by occluding the SMA (63). Using the SMA, the investigators then reperfused the organ with either blood, perfluorochemical (PFC) oxygenated at 20%, or leukocyte-enriched PFC. Histology revealed that mucosal injury after IR in the blood and PFC plus leukocyte groups was similar and significantly more extensive than the PFC alone group and non-reperfused controls. Clearly, neutrophils contribute substantially to the pathophysiology of IR injury.

Arachidonic Acid Metabolites

Although OFRs have a central role in the influx of neutrophils into the intestinal mucosa, they do not act as direct neutrophil chemoattractants. Instead, OFRs trigger the generation of the potent chemoattractants, LTB_4 and PAF (64, 65). Arachidonic acid formation occurs in the lipid membrane following phospholipase A_2 activation, a result of OFR interaction with mucosal endothelial cell membranes (49). Phospholipase A_2 also

converts membrane phospholipids to platelet activating factor (27). Leukotrienes, including LTB_4, are the product of arachidonic acid metabolism by the lipoxygenase pathway (66). The cyclooxygenase pathway of arachidonic acid metabolism forms thromboxanes (A_2 and B_2) and prostaglandins (66).

Neutrophils express specific cell surface receptors for LTB_4. Binding of LTB_4 to these receptors causes production and activation of CD18, an adhesion molecule, and OFR and protein synthesis (66). Ischemia alone fails to cause significant increases in mucosal LTB_4 levels; however, subsequent reperfusion of the intestine results in drastic, significant elevations in LTB_4 production from pre-ischemia baseline measures (43, 67). The degree of mucosal injury and neutrophil influx correlated with increased LTB_4 levels. Further supporting the role of LTB_4 in IR injury, LTB_4 receptor antagonist pretreatment inhibited IR-induced increases of LTB_4 and lessened neutrophil infiltration (68, 69), and significantly improved survival in rats after 90 minutes of ischemia and 2 hours of reperfusion (69).

PAF and thromboxane A_2 are additional arachidonic acid metabolites that contribute to the damage incurred during IR. Neutrophil adhesion and platelet aggregation and activation are triggered by PAF (27). Thromboxane A_2 is a powerful vasoconstrictor and trigger for platelet aggregation (27).

Inhibition of Nitric Oxide

Nitric oxide (NO) is a potent vasodilator. It has protective properties in the setting of IR injury, but is unfortunately inactivated by an OFR generated during IR, superoxide (70,71). Both dysfunctional release and inactivation of NO, leads to excessive vasoconstriction in the microvasculature of the intestinal mucosa (71,72). Kubes

administered N^G-nitro-L-arginine methyl ester (L-NAME) to inhibit NO synthesis in a model of IR (73). Compared to IR alone, blockade of NO production resulted in greater mucosal vascular permeability after 90 minutes of ischemia and 1 hour of reperfusion. Interestingly, both the severe nature of mucosal damage and the increased permeability in the L-NAME treated tissues were improved by L-arginine delivery prior to reperfusion. Additional external sources of NO, such as nitroprusside were also shown to lessen the IR-induced changes in mucosal and microvascular permeability (74).

The No Reflow Phenomenon

One would expect intestinal blood flow to return to pre-ischemic rates after reperfusion. Counter intuitively however, research has shown that blood flow through the intestine actually decreases following reperfusion (43,74,75), and can even continue to decline (43,75). Because of the observation that systemic mean arterial pressure is not significantly altered after reperfusion, an additional factor must underlie the reduction in blood flow (35,41). This additional factor has been termed the “no reflow” phenomenon, and contributes substantially to intestinal injury following IR. Using a rat intestinal transplant model, Massberg *et al* performed *in vivo* fluorescent microscopy to image the small bowel microcirculation (35). Tissues were cold stored between 6 and 24 hours prior to reperfusion. Microscopy revealed that the number of villi perfused decreased in a proportional manner to the time of cold ischemia. Control animals had 100% of the villi perfused; whereas, the number of villi perfused plummeted to 40% in tissues stored for 24 hours. The 6, 12, and 18-hour preservation groups had 99.8%, 89.7%, and 87.9% villi perfused, respectively. Mucosal red blood cell (RBC) velocity and functional capillary density decreased with increasing preservation times, as did

muscular microcirculation, a finding that correlated to rolling and adherent leukocyte numbers in submucosal venules. Other investigators subjected intestinal tissue to *in vivo* ischemia for 30 minutes followed by a reperfusion period of 1 hour (60). Compared to sham-operated controls, experimental animals had significantly lower RBC velocity in submucosal arterioles and venules, which decreased to the point of no reflow within the 1 hour of reperfusion. Again, leukocytes were implicated, as their rolling and adhesion within vessel walls correlated with reduced microcirculatory blood flow and greater mucosal injury. Together, these studies document not only the “no reflow” phenomenon in the intestine, but also the importance of neutrophils to the pathophysiology of “no reflow.” As discussed above, OFRs stimulate neutrophil migration and activation. Contributing to decreased blood flow after reperfusion, activated neutrophils increase capillary fluid leak into the interstitium by enzymatic degradation of the endothelial lining and basement membrane. They also provide a mechanical barrier to blood flow by accumulating within capillaries.

The Consequences of Ischemia-Reperfusion Injury

The mediators of IR injury, OFRs and neutrophils, have devastating effects on the structural integrity of the mucosal barrier, resulting in abnormal intestinal function. The extent of OFR generation and neutrophil infiltration correlates to the severity of intestinal damage (67).

A variety of parameters are used to assess the post-IR functioning of the small intestine. One measure of injury after IR is microvascular permeability. The lactulose/mannitol permeability test compares lactulose and mannitol urine concentrations after injection of a known quantity of each sugar. When compared to

controls, an increased urine lactulose to mannitol ratio was observed in dogs following transplantation, indicating increased permeability (76). Studying small bowel mannitol flux in Ussing chambers, other investigators showed that longer hypothermic preservation periods correlate with greater mucosal permeability and more extensive mucosal damage on histology (38). Another measure of intestinal permeability is the estimated osmotic reflection coefficient (σ_D). Calculated by the ratio of lymphatic to plasma protein concentrations, the σ_D correlates with neutrophil infiltration (59), histologic mucosal damage (42), and OFR generation (57,77).

An additional, highly critical function of the intestine is to provide a physical barrier against the translocation of microbes. Indeed, intestinal transplant patients have a heightened risk of sepsis and its complications (78). One study showed that 81% of the patients who received multivisceral transplantation and developed sepsis had bacterial isolates in the blood that were identical to those in the stool (79). In a retrospective review looking at nine years of intestinal transplants in children, patients had a mean of 2.1 bacteremia episodes (80). These studies reflect the reduced capacity of the small bowel to protect against bacterial translocation after transplantation of the small intestine.

The mucosal activity of glutaminase and maltase, and intestinal lipid absorption have been used as indicators of intestinal function post-IR. Conversion of glutamine to glutamate and ammonium is carried out by glutaminase (81). Mature enterocytes contain the enzyme maltase within their brush boarder, which is responsible for digesting maltose to glucose (82). Although only moderate decreases in maltase activity are seen after 24-hour intestinal cold-storage, greater reductions are observed upon reperfusion (39,83). Multiple other reports have shown that glutaminase activity at 20

minutes of reperfusion, following 1 to 2 hours of cold preservation, is highly predictive of the severity of IR injury (36,84,85). Kurtel *et al* examined small bowel function by using lipid absorption as a marker (86). They found that after delivery of a lipid-concentrated meal 24 hours post SMA occlusion for 10 minutes, lymphatic lipid transport was significantly reduced. Neutrophils were implicated in the altered lipid transport, since neutropenic animals failed to exhibit mucosal lipid transport dysfunction.

Principles of Organ Preservation

The induction of hypothermia is a necessary component of effective organ preservation (87). Van't Hoff's rule states that metabolic rate decreases 12-13 times when a tissue cools from 37 to 0°C. Thus, hypothermia prolongs preservation times by decreasing the rate of enzymatic degradation. Metabolism does not entirely cease, however, meaning that cooling cannot effectively preserve an organ alone. Preservation solutions are crucial for successful graft preservation. Although the specific formulation of different solutions varies, they all contain components that address the following issues: deleted energy substrates, cell swelling and edema, intracellular acidosis, and oxidative stress (87).

Hypothermia contributes to the preservation of ATP stores, but as metabolic inhibition is not complete, preservation solutions must supply substrates for ATP regeneration, allowing ATP-dependent processes to continue (87). Under anaerobic conditions, amino acid transamination in the mitochondria of muscle, liver, and kidney releases energy. Amino acids with a high energy capacity, such as glutamic acid, could potentially replete ATP stores to a minimum level required for ATP-dependent systems

to function. Some preservation solutions contain adenosine as an energy source. Stimulating post-perfusion ATP synthesis, adenosine can restore high-energy phosphate levels.

As previously discussed, cell swelling and edema are consequences of hypothermic ischemia. To reduce transmembrane water flux, preservation solutions eliminate the transmembrane Na^+/K^+ gradient (87). 'Intracellular-like preservation solutions' achieve this by mimicking the intracellular environment by providing K^+ and Na^+ at concentrations near that of the cytosol ($[K^+]$ = 100 – 130 mM and $[Na^+]$ = 10 – 30 mM). A consequence of high K^+ concentrations, these solutions cause blood vessel constriction, unfortunately. Extracellular-like preservation solutions also lessen cellular edema. The intracellular colloidal osmotic pressure is counterbalanced by an external osmotic pressure generated by a high Na^+ concentration.

'Impermeant' molecules are another important component of preservation solutions (87). These molecules are required to help neutralize cell membrane water shifts; Na^+ and K^+ concentration adjustments are not sufficient. Mannitol (181 Da), raffinose (504 Da), glucose (180 Da), lactobionic acid (357 Da), and gluconate (195 Da) are some examples of impermeant molecules. The concentration of impermeants must be near that of the osmotically active intracellular molecules (110-140 mM) to exert a pressure which counterbalances the physiological intracellular force.

At the time of organ harvest, when the organ is washed out and perfused with preservation liquid, contents of the solution diffuse into the interstisium and cause edema (87). 'Colloids' are macromolecules that increase the oncotic pressure of the preservation solution, reducing interstitial swelling. Albumin (70 kDa), hydroxyethyl

starch (HES, 250 kDa), polyethylene glycol (PEG, 20-35 kDa), and dextran 40 (40 kDa) are some colloids found in various solutions.

The cold-storage solution must also protect against increased intracellular Ca^{2+} concentrations, which can result in cell death (87). Preservation solutions should have low Ca^{2+}, high Na^{+}, and cautiously adjusted K^{+} and Mg^{2+} concentrations. Low Ca^{2+} concentration prevents the passive diffusion of Ca^{2+} into the intracellular space. Entry of Ca^{2+} through the Na^{+}/Ca^{2+} exchanger is reduced by a high Na^{+} concentration. To achieve decreased Ca^{2+} influx, Mg^{2+} concentrations are high; Mg^{2+} and Ca^{2+} compete for cellular entry. A high Mg^{2+} concentration also ensures intracellular retention of this ion, limiting Ca^{2+} release form organelles. Voltage-gated Ca^{2+} channels are prevented from opening by a low K^{+} concentration.

Buffering agents, which combat the development of intracellular acidosis that develops during cold ischemia, are another important property of preservation solutions (87). Commonly used buffers include bicarbonates, phosphate, citrate, HEPES, histidine, and tryptophan. The ability to transverse the cell membrane and to function at hypothermia are both important properties of buffers used for cold-storage.

Reactive oxygen species (ROS) are generated through a variety of complex cellular pathways during ischemic cold-storage and subsequent reperfusion of an organ. The deleterious effects of these molecules and the saturation of physiological antioxidant defences necessitates that preservation solutions contain antioxidant therapies or ROS scavengers (87). ROS scavengers in storage solutions are especially important within the extracellular space, where antioxidant abilities are relatively less. The cells themselves have intracellular enzymes, such as superoxide dismutase, catalase,

glutathione peroxidase and heme oxygenase-1 that provide some protection against ROS damage.

Allopurinol and reduced glutathione (GSH) have been used to prevent ROS-induced damage (1). Unfortunately, the mechanism of action of allopurinol during ischemia has yet to be fully elucidated. However, oxidation of GSH to GSSG, a reaction catalyzed by glutathione peroxidase, is a central mechanism in the detoxification of ROS.

Properties of Preservation Solutions

Electrolyte Composition

An intracellular type preservation solution, the original formulation of the UW solution had high potassium and low sodium concentrations to control edema (88). Though, it was later demonstrated that more effective maintenance of the Donnan equilibrium is achieved through extracellular like solutions (high sodium and low potassium). Also, due to the potassium induced vasospasm that accompanies intracellular like solutions, extracellular solutions are superior in terms of blood washout (89). One extracellular type solution is Celsior (88). It resembles UW solution except for the impermeant mannitol, histidine buffer, and the absence of a colloid. The composition of histidine tryptophan ketoglutarate (HTK) solution differs more from UW than Celsior does (88). Like Celsior, HTK is an extracellular like solution, and contains the impermeant mannitol and the buffer histidine. In addition, HTK employs the amino acids tryptophan, for its anti-oxidant and membrane-stabilizing properties, and ketoglutarate, a substrate for anaerobic metabolism. Polysol is a new and complex extracellular like solution (88). It contains the impermeants raffinose and gluconate, polyethylene glycol

(PEG) as the colloid, the buffers phosphate, histidine, and HEPES, a number of different ROS scavengers, 21 different amino acids, and 16 different vitamins.

Impermeants and Colloids

Impermeants and colloids are essential components of hypothermic preservation solutions, helping to counteract tissue edema (88). Both the UW and Celsior solutions include the anionic impermeant lactobionate. A large body of evidence supports the important role lactobionate plays in counteracting damaging fluid shifts that occur during intestinal preservation. The UW solution also contains the trisaccharide impermeant raffinose, whereas Celsior includes the monosaccharide mannitol. Dialysed HES provides colloid support in UW solution, but the accompanying increase in viscosity and potential for red blood cell aggregation has negative effects on wash-out and reperfusion. Alternative colloid agents, such as dextrans and PEG, have been implemented as a result. Neither colloid is included in clinically used solutions at the moment, however.

Buffers

Numerous buffers are utilized by cold-storage solutions to maintain homeostatic pH levels (88). A phosphate buffer is included in UW solution, while HTK relies on histidine. Three buffering agents are contained in Polysol: a phosphate buffer, histidine, and the sulfonic buffer HEPES. Unfortunately, the true value of the various buffers within cold-storage solutions is unknown, as no single study has assessed the association between pH fluctuations and outcomes following intestinal transplantation.

Anti-oxidants

Various ROS scavengers are included in preservation solutions to mitigate the injury mediated by ROS during cold storage and eventual reperfusion (88). The anti-oxidants within the UW solution are allopurinol and reduced glutathione. Celsior contains only glutathione. Allopurinol exerts its effects by blocking the enzyme xanthine oxidase. Glutathione converts damaging peroxides as it becomes oxidized. Full prevention of oxidative stress does not occur, however, at low concentrations (UW solution) of allopurinol and glutathione. A component of HTK solution, tryptophan has electron-accepting metabolites that scavenge ROS (88). Polysol contains a combination of different antioxidants. Reduced oxidative stress and improved graft quality has been documented using high concentrations of histidine in addition to glutathione/tryptophan, and by using a combination of antioxidants, as with Polysol.

Amino Acids

Amino acids are ATP precursors and are added to storage solutions, serving as substrates to quickly regenerate cellular energy levels (88). During preservation, their presence facilitates metabolic and synthetic cellular processes. Glutamine, the enterocyte's primary energy source, has shown benefits for both intravascular and intraluminal preservation. Polysol contains 21 different amino acids, but the exact mechanisms and the separate values of the amino acids have yet to be determined.

Intravascular Preservation of the Small Intestine

Currently, the small intestine is harvested as part of a multi-organ procurement protocol, involving an intra-aortic vascular flush with the 'gold standard' preservation

solution. The solution used as the ‘gold standard’ can differ between institutions. UW solution (Table 1-1) still remains the most commonly used solution for procurement of the intra-abdominal organs, but more recently, many centres are using HTK solution. These intravascular solutions, as well as others, have proven efficacy for static storage of the liver, kidneys, and pancreas. Regardless of the intravascular solution employed, however, no one solution has ever proven truly effective for static cold storage of the small intestine.

Components	UW
HES (g/L)	50
Lactobionate	100
Raffinose	30
KH_2PO_4	25
Allopurniol	1
Glutathion	3
Adenosine	5
Magnesium-sulfate	5
Chloride	20
Potassium	120
Sodium	25

Table 1-1. Composition of UW solution.
Units in mmol/l unless noted otherwise.

In an early comparison between UW, EC, and Lactated Ringer’s (LR) solution, EC solution proved most effective (90). Following 24 hours cold storage and subsequent autotransplantation, canine small intestines preserved with EC solution showed significantly less lipid peroxidation and neutrophil infiltration, as assessed by malondialdehyde and myeloperoxidase levels, respectively. A more recent communication also documented the protective effect of EC solution on the mucosal epithelium during cold storage, though RL had an even greater protective effect (91). Irreversible, crypt layer damage had occurred by 12 hours in tissues stored in either

solution, however. Contrasting previous findings, preservation with UW solution proved superior to EC solution for static cold storage of Lewis rat Jejunum (92). Rats underwent syngeneic heterotopic transplantation after 24 or 48 hours preservation with EC or UW solution. Survival rates were significantly higher in the UW groups at both storage time points, and the 24 hour UW group had the best maintenance of neural activity. Further complicating the matter, 7 hour cold ischemic storage in either UW or EC solutions both allowed for effective regeneration of ATP levels (93). Rat intestines preserved with a simple solution, Krebs-Henseleit-bicarbonate buffer (KHB), recovered ATP values to just 60% of controls. Near complete regeneration of ATP was observed in tissues stored with UW or EC solutions. Similar results were seen for energy charge. Predating these findings, tissues stored in UW and EC solutions had superior biochemical profiles compared to intestine stored in normal saline (94). At 6 and 24 hours storage, grafts preserved with both UW and EC solutions had significantly higher ATP content than saline treated tissues.

One extensive study compared the effects of EC, UW, and albumin-dextran-adenosine-allopurinol-verapamil (ADAAV) solutions on 24 hour hypothermic preservation of rat small intestine (95).Three hundred and forty-eight isogeneic Sprague-Dawley rats were used as donors and recipients. Early (3 days) post-transplant survival in the ADAAV group was not significantly different from saline controls (no storage), suggesting an early survival advantage. None of the solutions, however, demonstrated superiority over the long term (> 3 days). Functional and histologic data failed to show significant differences between groups at any time.

Another research group compared seven different storage solutions to saline: EC, UW, HTK, phosphate-buffered sucrose (PBS 140), extracellular fluid (ECF),

lactobionate fructose, and modified lactobionate fructose (96). Following 12 hour small bowel preservation, 7-day post-transplant survival rates failed to show any significant differences between groups.

After documented success in the realm of heart preservation, Celsior was yet another solution employed to store the small intestine (97). This in vitro study subjected rat grafts to 18 hour cold-storage in Celsior or UW solution. While Celsior demonstrated superior vascular perfusion characteristics and more favorable enzyme release and carbohydrate absorption, there were no significant differences regarding histology, edema formation, energy charge, or creatine phosphate.

DeRoover et al. compared the effect of UW and Celsior solutions on human intestinal preservation (98). Similar to the previous animal model, no significant differences in histology were seen throughout the 24 hour storage period. In both groups, epithelial detachment began early at 6 hours of preservation and progressed to crypt damage by 24 hours.

Moving from in vitro human tissues to clinical transplant outcomes, Mangus et al. assessed graft and patient survival, early graft function, and rejection (99). Between 2003 and 2007, 54 patients underwent intestinal transplantation with 57 grafts (includes multivisceral grafts). Twenty-two were preserved with UW and 37 with HTK. Immunosuppression protocols were similar between groups. Analysis of grafts stored in either HTK or UW revealed no significant difference in graft or patient survival at 30 and 90 days post-transplant. Further, there were no differences in endoscopic appearance, initial function, number of rejection episodes, or transplant pancreatitis.

Intraluminal Preservation of the Small Intestine

Given the non-success of any single intravascular solution to optimally preserve the small bowel, some researchers started looking to the intestinal lumen as an additional route to deliver preservation solution. Common intravascular solutions, as well as intestinal specific solutions, have been investigated as intraluminal flushing agents. Perhaps the greatest advantage to intraluminal delivery of preservation solution is that one can specifically tailor the solution to the intestine without affecting procurement of the other intra-abdominal organs.

In one of the earliest studies suggesting a benefit to luminal irrigation of the intestinal graft, Raju et al. were determined to extend cold storage time beyond the current experimental standard of five hours (100). Canine grafts received both an intravascular and an intraluminal flush with RL solution. After either 12 or 24 hours hypothermic storage in RL, intestines were allotransplanted into recipients. All animals survived past 5 days in the 12 hour storage group, and 67% survived past 5 days in the 24 hour storage group. This level of success had never been achieved in the setting of extended storage time, and luminal irrigation was one of the technical factors identified as contributing to the success.

The UW solution was another intravascular agent tested on the intestinal lumen. After intravascular and intraluminal irrigation with UW solution and 18 hours cold storage, however, only rat tissues receiving luminal gas oxygenation during storage maintained metabolic status and absorption abilities (101). Zhu et al. also showed that luminal gas oxygenation with UW solution had positive effects on energy levels, oxidative stress, and mucosal injury during ischemic cold storage of rat intestine (102).

More recently, histological analysis of human tissues showed that luminal contact with UW solution, in addition to a UW intravascular flush, better preserved intestinal morphology than the clinical standard of a UW intravascular flush alone (103). The degree of damage to the intestinal mucosa was significantly lower at each time point in the luminal treated tissues compared to controls. Soon after these results, Leuvenink et al. reported that luminal delivery of UW, UW with glutamine, or Celsior could not prevent severe damage to the intestinal mucosa in rats (104). Failure to include an intravascular flush as part of the procurement protocol could provide a reason for the poor mucosal preservation in all three groups, highlighting the importance of using luminal preservation in addition to the standard intravascular method.

In an effort to evaluate the newly developed Polysol solution for preservation of small intestine, Wei et al. compared Polysol to the standard solutions, UW, HTK, and Celsior (105). Rat intestines were flushed by intravascular and intraluminal routes, harvested, and then cold-stored for 18 hours in their respective solutions. Subsequent reperfusion with oxygenated Krebs-Henseleit buffer at body temperature for 30 minutes followed. The highest ATP levels were seen in the Polysol group, which also had the lowest lactate dehydrogenase release. Although, Celsior and HTK also had high ATP content and low LDH release compared to UW solution. UW solution also fared the worst in terms of lipid peroxidation and apoptosis, having significantly higher levels of both parameters than each of the other groups. Electron microscopy revealed superior maintenance of microvilli and mitochondria architecture in the Polysol group. The findings presented in this study highlight the potential of Polysol solution in the cold storage of small intestine, but more studies are needed to fully investigate its effects.

Instead of experimenting with luminal delivery of previously developed intravascular preservation solutions, some researchers began developing intestine-specific, luminal preservation solutions. Unlike intravascular solutions, which flow through all intra-abdominal organs at time of organ harvest, there is an opportunity to tailor intraluminal solutions to the requirements of the intestine. In 2002, Fujimoto et al. began doing just that (38). They found that luminal delivery of, and storage in, an amino acid (AA) - based preservation solution (Table 1-2) maintained the most optimal *in vitro* preservation parameters. Luminal UW and luminal UW with glutamine were the other two experimental groups, and the control was the clinical standard of a UW vascular flush alone. The AA solution contained 19 amino acids, all of which are postulated to be important to mucosal functioning. Two variations of AA solution were tested (AA1 and AA2). The important differences being the inclusion of an osmotic agent (lactobionate) and BES buffer in AA2. In terms of energy status, barrier function, and histology, tissues flushed and cold-stored in AA2 were better preserved than any other group. One follow-up study noted that although the AA solution ameliorates much of the mucosal damage during cold storage, there still remains a degree of barrier function disruption. In an effort to reduce bacteria counts, and thus potential bacterial translocation, Olson et al. added a luminal povidone-iodine flush before the AA solution flush (106). Unfortunately, luminal povidone-iodine exposure did not significantly affect bacteria counts, and actually potentiated preservation injury.

Components	AA
Lactobionate	20
Colloid (g/L)	50
BES	15
Glucose	20
Adenosine	5
Hydroxybutyrate	3
Trolox	1
Allopurniol	1
Gln	35
Glu	20
Asp	20
Asn	10
Arg	10
Gly	10
Ser	10
Thr	10
Lys	10
Val	10
Pro	5
Cys	5
Met	5
Leu	5
Ile	5
His	5
Orn	5
Tyr	1
Trp	1

Table 1-2. Current composition of the AA solution.
Units in mmol/l unless noted otherwise.

Given the preliminary success of luminal AA solution in a rat model of cold storage, investigators tested its effects on human tissues (107). The results were not as promising, however. Both UW and AA luminal treatments had significantly better mucosal barrier function (assessed by manitol permeability) than the UW vascular group, but no differences in energy status were observed between any groups. More, histological damage was not affected by AA solution, and only marginally affected in the

luminal UW group. One of the suggestions for the poor performance of the AA solution was that the solution needed additional osmotic support (lactobionate was not sufficient). One major change to the AA solution at this point on was the addition of dextran as an oncotic agent.

After additional success in a rodent model of continuous perfusion (108), as well as cavitary two-layer preservation with perfluorocarbon (109), the AA solution was tested in an *in vivo* rat model of ischemia reperfusion (IR) injury (110). Tissues treated with luminal AA solution prior to 60 minute warm ischemia had superior energy levels, amino acid utilization, barrier function, morphology and less oxidative stress, and neutrophil recruitment during 60 minute reperfusion, compared to non-treated controls or the luminal RL group.

The AA solution was also evaluated using a rodent model of orthotopic small bowel transplantation (111). All donor rats received a UW vascular flush before grafts were harvested and flushed intraluminally with either UW or AA solution (control= no luminal flush). Tissues were cold stored for 6 hours before recipients underwent syngeneic transplantation. Survival was the most striking outcome measure; no animals in the UW or control groups survived past 12 hours post-transplant, while 80% survived until 14 days in the AA group. Biochemical endpoints and histology reflected the survival data. Wanting to understand the key factors contributing to improved graft quality after storage in AA solution, Salehi et al. investigated the link between stress responses and kinase signaling during graft preservation (112). They found that treatment with AA solution down-regulates the pre-apoptotic signals JNK and P38, and up-regulates the cytoprotective signal ERK.

Using a similar model of IR injury previously used in rats (110), researchers evaluated the ability of AA solution to prevent intestinal IR injury in pigs (113). Intestines were pretreated with luminal UW solution, AA solution, or nothing (control). Intestinal reperfusion for 180 minutes followed 60 minute *in vivo* ischemia. The most impressive finding was that AA solution treatment suppressed activation of AMP-activated protein kinase (AMPK), leading to the down-regulation of the proapoptotic kinase, Jun kinase.

Although the most extensively studied, the AA solution is not the only tailored intraluminal solution under development. Roskott et al. showed that intraluminal delivery of Williams Medium E with added buffer, impermeants, and a colloid (WMEplus), can reduce IR injury in cold - stored rat intestine (114). Amino acids and vitamins are major components of WMEplus, and the colloid employed is polyethylene glycol (PEG; Mw= 35 kDa). Polyethylene glycol was also the colloid contained in a low-sodium electrolyte solution that reduced intestinal preservation injury (115). The same research group most recently found that an intraluminal, low sodium PEG solution improves a number of epithelial characteristics during cold storage of rat small bowel; thus, enhancing graft viability (116).

Tailored intraluminal preservation solutions are a promising, relatively novel concept for the application of intestinal preservation. However, clinical studies using human tissues are needed to elucidate their true impact.

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Chapter 2: Comparing the effects of dextran 70 and hydroxyethylstarch in an intestinal storage solution[1]

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Introduction

Small bowel transplants have increased in frequency and success. In 2003, 140 transplant procedures were performed, compared to just 11 in 1990 (1); currently ~200 intestinal transplants are performed worldwide yearly. For those patients receiving antibody-based pretreatment therapy and tacrolimus-based immunosuppressants, one year post-transplant survival rates have matched those of the liver (>80%). However, long-term survival rates (5 years) continue to remain low, although there is much variability depending on type of immunosuppressive protocol (1).

Sepsis poses the major threat to successful intestinal transplantation, accounting for 46% of mortality (1). This complication may result from bacterial translocation following the disruption of intestinal barrier function, a consequence of inadequate preservation (2, 3). Since its formulation, the University of Wisconsin (UW) solution has been recognized as one of the 'gold standards' for the preservation of intra-abdominal organs. But despite its efficacy with other intra-abdominal organs, some report that UW solution performs on par or below that of other solutions such as normal saline when used for small bowel preservation (4, 5). Indeed, no one preservation solution has consistently proved superior over any other for intestinal storage (6-8). It is not

[1] *A version of this chapter has been published. Kokotilo et al. 2010. Cryobiology. 61: 254-262.*

completely unexpected that clinical storage times for the intestine remain relatively short (6-10 h) with highly varying levels of storage injury (9).

Recognizing the urgent need for a more effective intestinal-specific preservation solution, our laboratory has been developing an amino acid-based nutrient-rich solution tailored to the metabolic requirements of the small intestine. Unlike UW solution, the nutrient solution is administered to the intestinal lumen instead of via the intravascular route. A number of *in vitro* and *in vivo* studies have documented improved metabolic parameters, tissue morphology, and functional characteristics following various periods of organ storage in this nutrient-rich solution (10-12). Although the composition of both the UW and nutrient solutions differ in many respects, each contains molecules for impermeant support. Such agents assist in controlling hypothermia-induced edema, one of the primary requirements for effective organ preservation (13).

Over the past several years, as we have been developing our nutrient-rich intraluminal preservation solution, either dextran 70 (70 kDa) or hydroxyethylstarch (HES) has been included for the purpose of providing oncotic support (10-12, 14, 15). However, molecular weight characteristics of an appropriate agent have not been established. As the intestinal barrier function becomes compromised during ischemic cold-storage, some seemingly 'high' molecular weight compounds may be able to penetrate the mucosa to an appreciable degree due to disruption of mucosal barrier integrity. The current study aimed to clarify issues of increased mucosal permeability during ischemia and the characteristics of a truly impermeant molecule utilized for cold storage of the intestine.

Materials and Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada). Dextran 70 (D70) and hydroxyethylstarch (HES) had average molecular weights of 70 and 2,200 kDa, respectively. Dextran (catalogue number D4751) was purchased from Sigma; HES was purchased from Aldrich #465143, 25,000 cps and purified as described previously to produce a purer molecular weight fraction (15).

Stability of Fluorescent label and Starches

FITC Labeling Dextrans: The procedure used to FITC-label D70, HES and the starch in UW solution (UW-HES) was based on the original procedure used by De Belder and Granath (16). The molecule of interest, along with Fluorescein 5-isothiocyanate (FITC), was dissolved in dimethyl sulphoxide, pyridine, and dibutyltin dilaurate and heated to 95°C for 2 hours. The mixture was then put on ice and D70, HES or UW-HES was precipitated out with cold 100% ethanol, filtered, and dried.

Evaluating Stability of the Fluorescent Label on Dextran and HES: 'Fluorescence units per mg' values for FITC-D70 and FITC-HES were determined to establish purity and efficiency of the labeling process. A mass of 10-15mg was dissolved in Tris buffer (50 mM; pH 8.0). Appropriate dilutions were implemented and fluorescence was measured at Gain 60 on the Perkin Elmer HTS 7000 using the same excitation wavelengths as described above. Labeled D70 and HES solutions were incubated at room temperature

(22±2 ^{o}C) for 24 hours. Samples were precipitated with cold 100% ethanol, centrifuged, diluted in Tris buffer and fluorescence measured.

Evaluating the Stability of the Starches following the Labeling Procedure: For HES, D70 and UW-HES, a series of molecular weight fractions were determined using centrifugal filters (Microcon Centrifugal Devices) with varying molecular weight cutoffs. A 100mg/L solution of labeled starch and a 0.2g/L solution of unlabelled starch were spun through 100 000, 50 000, 30 000 and 10 000Da MWCO filters. The filtrates of the starches were analyzed using an anthrone-based starch assay. Sample was added to anthrone and sulfuric acid, heated for 3 minutes at 90-95°C, cooled on ice, plated and analyzed spectrophotometrically at 600nm. The filtrate measured with each of the MWCOs was compared to an unfiltered sample and the percentage of starch present was determined.

Permeability of Bowel to D70 and HES During Cold Storage

Surgical Procedure and Procurement of the Small Intestine: All experiments were conducted in accordance with Canadian Council on Animal Care policies. Rats were fasted approximately 10-12 h. Rats were anesthetized with inhalational isoflurane (0.5-2%) in oxygen followed by an intramuscular injection of pentobarbital (65mg/250g; IM). A midline incision was made exposing the aorta at the level of the celiac trunk. The supraceliac aorta was clamped and 2-4 mL of University of Wisconsin (UW) solution was administered through the infrarenal aorta. The suprahepatic vena cava was transected to allow the outflow of blood and UW solution. Following the intravascular flush,

experimental groups were designated according to the following intraluminal treatment:

UW: UW solution

AA-D70: nutrient solution with D70

AA-HES: nutrient solution with HES

Measurement of intraluminal dextran 70 or HES during cold storage: Separate groups of organs (n=4) were flushed and stored in AA-D70 and AA-HES; at the respective timepoints, a sample of intraluminal solution was extracted via a 23g needle and analyzed for starch (as glucose) (17). Since the solutions contained other sugars (glucose and lactobionate), it was necessary to prepare a calibration curve using a series of standards of known carbohydrates for use in the final calculation of starch contents (Figure 2-1).

<u>Organ Procurement Procedure</u>: Following intravascular and intraluminal flushes, the small bowel was harvested and 16 cm long sections of ileum were measured out. The sections were filled at the proximal end with 4-5 mL nutrient-rich preservation solution (termed the 'AA' solution due to a large component of amino acids) containing either FITC-labeled D70 or HES (10 X 10^6 Fluorescence Units/mL) and each end was ligated with 3-0 silk. The preservation solution contained (values in brackets are mM): Glutamine (35), Lactobionate (20), Glutamate (20), Aspartate (20), Glucose (20), BES [N,N-Bis(2-hydroxyethyl)taurine] (15), Arginine (10), Glycine (10), Asparagine (10), Threonine (10), Lysine (10), Valine (10), Serine (10), Methionine (5), Leucine (5), Isoleucine (5), Histidine (5), Ornithine (5), Proline (5), Adenosine (5), Cysteine (5), β-Hydroxybutyrate (3),

Tyrosine (1), Tryptophan (1), Trolox (1), 3-Aminobenzamide (1) , Allopurinol (1); plus the inclusion of 5% Dextran or HES. Solutions containing the respective starch were abbreviated AA-D70 or AA-HES. A control group using the University of Wisconsin (UW) solution as the intraluminal flush was included.

Tissues were stored on ice in a fridge (4°C) for a maximum of 12 h. At various times throughout a 12 h time course, short segments of bowel (2 cm) were removed from the distal end; the remaining segment of bowel was ligated to prevent escape of solution during the rest of the storage period. A segment was fixed in alcoholic formalin for fluorescence and light microscopy at 2 (fluorescence only), 4, 8, 12h. Alcohol was necessary in the formalin to precipitate the starches and to maintain localization of the FITC label. Another segment at 4, 8, 12h was immediately frozen in nitrogen and subsequently stored at -65 ^{o}C until processing for metabolite analysis. For fluorescence assessment, n=4; for metabolite analysis and H&E histology, n=6.

Histology: Bowel samples were fixed in alcoholic formalin solution, processed to paraffin wax, embedded, and sections cut 4-5μm thick. Slides were dewaxed up to 70% alcohol then mounted with Prolong Gold anti-fade reagent with DAPI (Invitrogen) and sealed with nail polish. Fluorescent microscopy was used to view FITC and DAPI fluorescence in tissue. Fluorescent images were digitally captured using AxioVision software. Ischemic injury was assessed using Park's grading system (18) with modification as indicated in Table 2-1.

Sample Preparation and Metabolite Assay: Frozen samples were extracted 1:5 w/v in perchloric acid containing 1mM EDTA. Immediately after homogenization in PCA, a 50 μl

aliquot was taken and transferred to 0.15 mM NaOH (950 µl) for solubilization prior to protein measurement for standardization of metabolite levels (19). The remainder of the acid homogenate was then centrifuged (20 min, 20,000xg) to remove precipitated protein. Acid extracts were neutralized with 3M KOH/ 0.4M Tris/0.3M KCl and recentrifuged (20min, 14,000xg). Neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (20). Spectrophotometric analysis was performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP. ATP values were reported as µmol per gram protein and Energy charge (EC) values were calculated as: EC=(ATP+0.5ADP)/(ATP+ADP+AMP). Malondialdehyde (MDA) was assessed from frozen tissue homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA (21).

Statistical Analysis: Data were reported as mean ± SE for each group. Statistical differences between groups were determined using Analysis of Variance (ANOVA), followed by SNK *post hoc* test; $p<0.05$ was reported. Histology grades were compared with a non-parametric Kruskal-Wallis test.

Results

Intraluminal Levels of Dextran and Hydroxyethylstarch Throughout Storage (Figure 2-1):

Following intraluminal flush with AA-dextran, levels of dextran (70 kDa) contained within the lumen remained at values not significantly different than 100% of initial throughout the first 2 h storage. However, by 4 h values dropped to 86 ± 4 % ($P<0.05$) and remained low and constant throughout the remainder of the 12 h time course ($P<0.05$). In

contrast, levels of hydroxyethylstarch remained at values not significantly different than 100% initial.

Stability of Labeled D70 and HES: The FITC label on both D70 and HES was stable; greater than 98.8% remained bound after 24 h at 22°C. Stability of the FITC label on the HES isolated from UW solution (UW-HES) was ~55%, with 45% of the FITC label liberating from the starch (data not shown).

Molecular Weight Cutoff Filters (Figure 2-2): This analysis was necessary to ensure the stability of D70 and HES for subsequent experimentation and to ensure validity of the interpretation of our results. According to the manufacturer monograph, the size of the molecules filtered is approximately 2 times greater than the filter specification (ie. the true molecular weight exclusion for a 100 kDa filter is ~200 kDa). Hence, discussion in this section will use filter cutoff values. Initially, we wanted to compare D70 with UW-HES; however, stability following the FITC-labeling procedure was extremely poor, indicating a considerable degradation (ranging from 24-50%) of the starch itself when exposed to heat ($P<0.05$ across all filter sizes). Due to the high instability of the UW-HES, it was excluded from any further experimentation. In contrast, both D70 and HES were much more stable as reflected in non-significant variations across the filter ranges.

Permeability to D70 and HES During Cold Ischemic Storage (Figure 2-3): During storage, D70 penetrated the tissue as early as 2 hours and continued to do so throughout the 12h storage period. Evidence of tissue damage throughout the time course was apparent. After 2h, small amounts of epithelial lifting were observed. By 12h, tissues

showed substantial damage; epithelial lifting was complete, and the mucosa was disintegrated. Contrasting these results, the HES remained impermanent to the small bowel over the duration of storage, residing within the lumen the entire time. Tissue morphology resembled that of near-normal bowel (with a minor degree of clefting) even after 12h storage.

Histologic Injury (Table 2-1; Figure 2-4): At 4 h and 12h of cold storage, only AA-HES treatment exhibited significantly improved injury scores compared to both UW and AA-D70 groups ($P<0.05$); median grades in the AA-HES group were 0 and 2, respectively, indicating normal tissue and moderate clefting along the villus sides. At 8 h, differences between grades for all groups were not significant different; median grades ranged from 2 to 3. After 12 h storage, median grades of injury in AA-D70 and UW groups were 4 and 6, respectively; these grades were indicative of completely denuded villi (grade 4) and infarction of the Crypts of Lieberkühn (grade 6). Representative regions of injury depicting median grades are presented in Figure 4.

Effect of D70 and HES on Energy Metabolism (Figure 2-5): Levels of ATP, AMP, and Energy Charge ratios exhibited a clear and distinct pattern throughout storage. The lowest levels for ATP and Energy Charge were in the UW group followed by preservation solution with D70 (AA-D70). The HES-containing solution (AA-HES) exhibited the highest levels of the three groups ($P<0.05$). Levels of the low energy adenylate, AMP, were elevated in the UW group at 4 h, but resumed initial t=0 values by 8 h; values in AA-D70 and AA-HES were significantly less than UW group. At all time points, AA-HES values were maintained at the lowest level of the three experimental groups.

<u>Effect of D70 and HES on metabolic end-products (Figure 2-6):</u> After 4 h, levels of anerobic end-product, lactate, rose by ~50% above initial (t=0) $P<0.05$; UW tissues exhibited no change. Values at 8 and 12 h were highest in the AA-HES group ($P<0.05$) followed by AA-D70 and UW groups. Products of amino acid metabolism, alanine and ammonium, exhibited varying degrees of accumulation with AA-HES maintaining the highest of the three groups by 8 h, $P<0.05$.

<u>Effect of D70 and HES on oxidative stress (Figure 2-7):</u> Levels of oxidative end-product, malondialdehyde (MDA), were highest in the UW treated group. Both AA-D70 and AA-HES groups showed excellent control of oxidative stress, as MDA levels remained low and constant throughout cold storage; $P<0.05$.

DISCUSSION

Dextrans are naturally occurring polymers of glucose produced by bacteria, the most well-known being of the *Leuconostoc* genus (22); hydroxyethyl starch (HES) is a semi-synthetic polymer of amylopectin (23). Both molecules are colloids whose major clinical application has been for their volume expanding properties. In past years, the use of dextrans has been faced with considerable scrutiny due to the occurrence of anaphylactoid reactions. As a result, dextrans are now synthesized in such a way as to reduce the number of side chains, decreasing the incidence of severe anaphylactoid reactions (23). Some reports even indicate that true anaphylactoid reactions resulting from dextrans occur with no significantly greater frequency than other colloids (24). Others indicate that the rate of HES induced anaphylactoid reactions (0.006%) is

remarkably lower than that of dextrans (0.273%) and other colloids (23). Molecular weight (MW) affects the colloidal activity of dextrans and HES (22). However, both of these colloids are polydisperse, meaning that a range of MW exists for any reported MW (23). HES is particularly polydisperse, containing individual colloid molecules anywhere from a few thousand to over several million daltons (21). MW can be characterized by a number of parameters including the 'weight average MW' (MWw) or 'number average MW' (MWn) (23). The former directly depends on the average of all molecular weight fractions present (and not the number of molecules in each fraction) and is thus highly influenced by unrepresentative numbers of very small or large molecules (23). On the other hand, the MWn represents a weighted average, reflecting the frequency of each individual colloid molecule (22). By calculating the ratio of MWw-to-MWn, the polydispersity index (PDI) is given, which indicates either low or high MW variability (higher PDI values indicate wider variability in molecular weight; whereas a PDI = 1.0 indicates a pure molecular weight fraction). An additional property of HES that influences its colloidal activity is the degree of substitution of the hydroxyethyl moiety (23). Native starches are rapidly hydrolyzed in the mammalian body, but the higher the degree of hydroxyethylation of the glucosyl units at the C2 position compared to C6, the slower the rate of starch degradation by endogenous enzymes (23). When selecting a colloidal agent such as HES, these parameters describing the agent must be considered in order to avoid a significant component of low molecular weight fractions.

Organ preservation solutions contain cell-impermeant molecules to counteract tissue edema during cold ischemic storage. A number of metabolic and physiologic changes accompany current storage strategies and contribute to the disruption of the mucosal barrier. Tight junctions, located near the luminal face of the epithelial cell layer,

maintain an effective barrier; they consist of large dynamic proteins that are dependent on cellular energy levels (10). Since energy-consuming processes still operate to some degree at low temperatures, energy levels rapidly decline, particularly in the face of restrictions on oxidative-phosphorylation during ischemia (10, 25). Consequently, tight junctions dilate and epithelial cells lose their ability to sustain a barrier, increasing the flux of intraluminal macromolecules across the mucosa (26). Transepithelial flux of enteric bacteria is of particular concern due to direct implications for life-threatening sepsis. At a molecular level, the Na+/K+ pump maintains transmembrane ion gradients, such that extracellular Na+ levels are high and K+ levels are low (intracellular concentrations are reversed) (27). ATP, produced primarily through oxidative phosphorylation, provides the energy required by the pump. Under normal conditions, energy levels are sustained, and Na+ is preferentially excluded from the intracellular space. The extracellular Na+ creates an osmotic pressure which counters the osmotic pressure exerted by impermeable anions and proteins. The force created by these intracellular sources has been calculated as 110-140 mOsm/kg. However, under conditions of cold ischemic storage, this delicate balance is disrupted. Collectively, hypothermia coupled with declining ATP levels suppress Na+/K+ pump function, thereby decreasing the cell membrane potential. In response to this charge disruption, Na+ and Cl- flow down their concentration gradients into the cell interior. As water follows the flux of ions, the cell begins to swell. If allowed to continue indefinitely, a loss of cell function and lysis of the cell membrane would occur. In order to offset the generated imbalance in osmotic forces, cell-impermeable agents can be utilized to maintain osmotic equilibrium. Clearly, the maintenance of cell/tissue viability involves energetic and osmotic components, where either component influences the other. In the current

communication, a metabolic attempt to generate ATP from both anerobic and aerobic processes was apparent. Within the first 4 h, all groups (including UW which contained no amino acid substrates) exhibited an accumulation of alanine and ammonium that would suggest catabolism of endogenous amino acids. However, at subsequent times (8 and 12 h) alanine was preferentially produced in tissues treated with the nutrient-rich solution (AA solution) supplemented with 5% hydroxydethylstarch, implicating the conversion of preservation solution amino acids. The relative contribution of anaerobic processes (ie. glycolysis) was also greatest in the hydroxyethylstarch containing solution. These findings reflect the involvement of both aerobic and anerobic metabolism in an attempt to replenish dwindling energy reserves. However, it is difficult to determine relative contributions of anaerobic versus aerobic metabolism to ATP synthesis since the rates of end-product transport out of the intestine during cold ischemia, either into the vasculature or into the lumen, are currently unknown.

In order for a preservation solution to be effective, it must provide an external osmotic force sufficient to counteract intracellular forces; thus, preventing shifts in water leading to edema. Without an intraluminal flush and storage solution, it is difficult to provide adequate osmotic support to the mucosa, although there would be some contribution via the intravascular route. In the past, intravascular preservation solutions have used low molecular weight compounds such as mannitol, lactobionate, raffinose, and gluconate as effective osmotic impermeants. However, in the realm of an intraluminal preservation solution, the agents must remain impermeant to the mucosa. Throughout the literature, the permeability of dextran (Mw= 4400 Da) has been used as a reliable indicator of the effects of intestinal ischemia on mucosal permeability in *in vivo* models (28). Furthermore, Ekstrom et al. failed to find appreciable amounts of

fluorescein-conjugated dextran 70 kDa in the serum or enterocytes after orogastric administration of the labeled dextran in healthy, post-closure rats, indicating that this molecular weight fraction is an effective impermeant when administered to the lumen of healthy rats (29). Over the last few years, we have included dextran 70, lactobionate, and HES in our solution as arbitrary choices based on previous literature for primarily vascular impermeants [10, 30, 31]; however, these choices were not supported by any direct evidence. The exact molecular weight that defines a molecule as 'impermeant' has not been elucidated for an intraluminal preservation solution during cold storage, a time when there is significant ischemic stress. During the cold storage time-course, in which D70 and HES were incorporated into the intraluminal preservation solution, direct visualization with fluorescent micrographs revealed that D70 fully penetrated the intestinal barrier as early as 2 h. Moreover, these tissues had developed extensive damage, with epithelial clefts developing after 2 h, and complete denudation of the villi by 12 h. In contrast, grafts stored in the presence of HES exhibited an intact mucosal barrier and retained an intact morphology throughout all time-points. Our lab has utilized this high Mw (2,200 kDa) starch in two recent studies (14, 15); the starch product has a presumably low degree of hydroyethyl-substitution (as implicated by poor solubility characteristics) and has a relatively moderate viscosity at 5% concentration (although we have not measured this). Nevertheless, with heating the starch solution prior to the addition of the other components, complete solubility is possible to 10%. As well, the elevated viscosity does not preclude effective delivery of the solution even at reduced temperatures (0-4°C). Although previous work by Nejdfors et al. concluded that there is an inverse relationship between permeability and molecular weight in multiple species, larger molecules > 70 kDa were not explored in the realm of intestinal

ischemia (32). Our study supports their conclusion and further implicates the need for a much larger molecular weight molecule (> 70 kDa) when employing an intraluminal preservation solution during cold storage.

Although our study focuses on the importance of an intraluminal preservation solution tailored to intestinal metabolism, preservation of the vasculature remains an important aspect of organ viability. Damage to the endothelium can elicit inflammatory/immune responses leading to injury to the entire organ, so both vascular and intraluminal strategies are vital to organ viability. We have developed a strategy of intraluminal preservation solution administration based on the fact that the confines of clinical practice dictate that a common intra-aortic flush with a standard intravascular preservation solution is performed as part of a multi-organ procurement procedure. Once all relevant organs have been flushed intravascularly to clear the blood and initiate tissue cooling, the intestine is removed, thereby permitting further treatment of the intestine on the 'back table'. In order for any change to current protocols to be instituted in the clinic, the proposed alteration must first be safe, and second, result in a substantial improvement in clinical outcomes; it is becoming apparent that our strategy of satisfies both criteria. Future studies will focus on translating the findings in this study to a small animal transplant model in order to demonstrate direct clinical applicability.

To summarize, the permeability characteristics of the small intestine change markedly under ischemic conditions, permitting even relatively high molecular weight molecules such as D70 to cross the mucosal barrier during cold ischemia. In contrast, a high molecular weight HES remained impermeant throughout 12 h cold storage and contributed to the preferential maintenance of energetic parameters and mucosal morphology. The generation of ATP from both anaerobic and aerobic pathways is

implicated, while at the same time minimizing oxidative stress. The results presented in this study support the use of HES (Mw=2,200 kdal) as an effective impermeant for an intraluminal preservation solution.

Tables and Figures

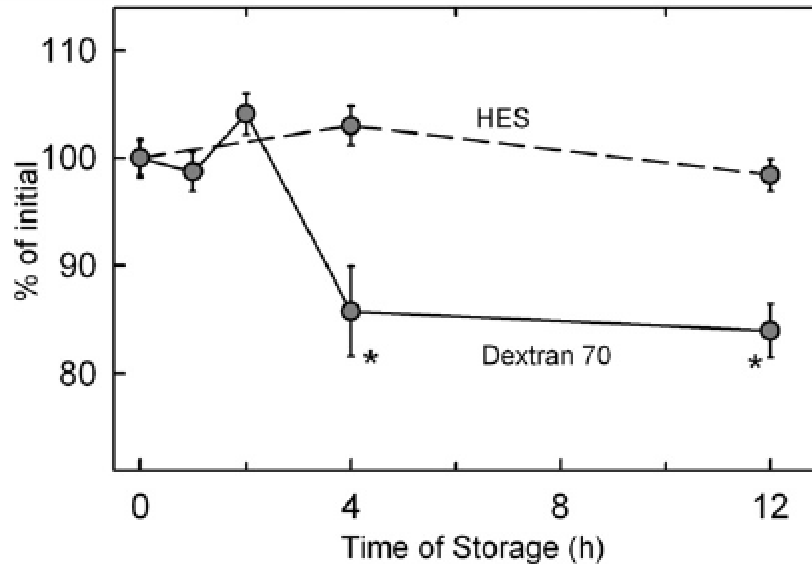

Figure 2-1. Intraluminal levels of Dextran and Hydroxyethylstarch throughout cold storage.
Dashed and solid lines indicate levels of HES and Dextran 70, respectively in the intraluminal solution during cold storage. * -denotes significantly different from 100% initial value; P<0.05.

	4 h	12 h
UW	1,1,2,3,3,3 (2.5)	3,3,6,6,6,7 (6)
AA-D70	1,2,2,2,3,3 (2)	3,3,4,4,4,4 (4)
AA-HES	0,0,0,0,1,1 (0) †‡	0,0,1,3,3,3 (2) †‡

Table 2-1. Histology Grades of Intestinal Injury following Cold Storage.
Values in brackets indicate median value. †, ‡ -Significantly different from UW and AA-D70 respectively; P<0.05. Grading of intestinal injury is based on Park's classification of intestinal injury (18).

0 = Normal mucosa; 1 = Subepithelial space at villus tip;
2 = Moderate subepithelial space; 3 = Epithelial lifting along villus sides;
4 = Denuded villi; 5 = Loss of villus tissue; 6 = Crypt layer infarction;
7 = Transmucosal infarction; 8 = Transmural infarction.

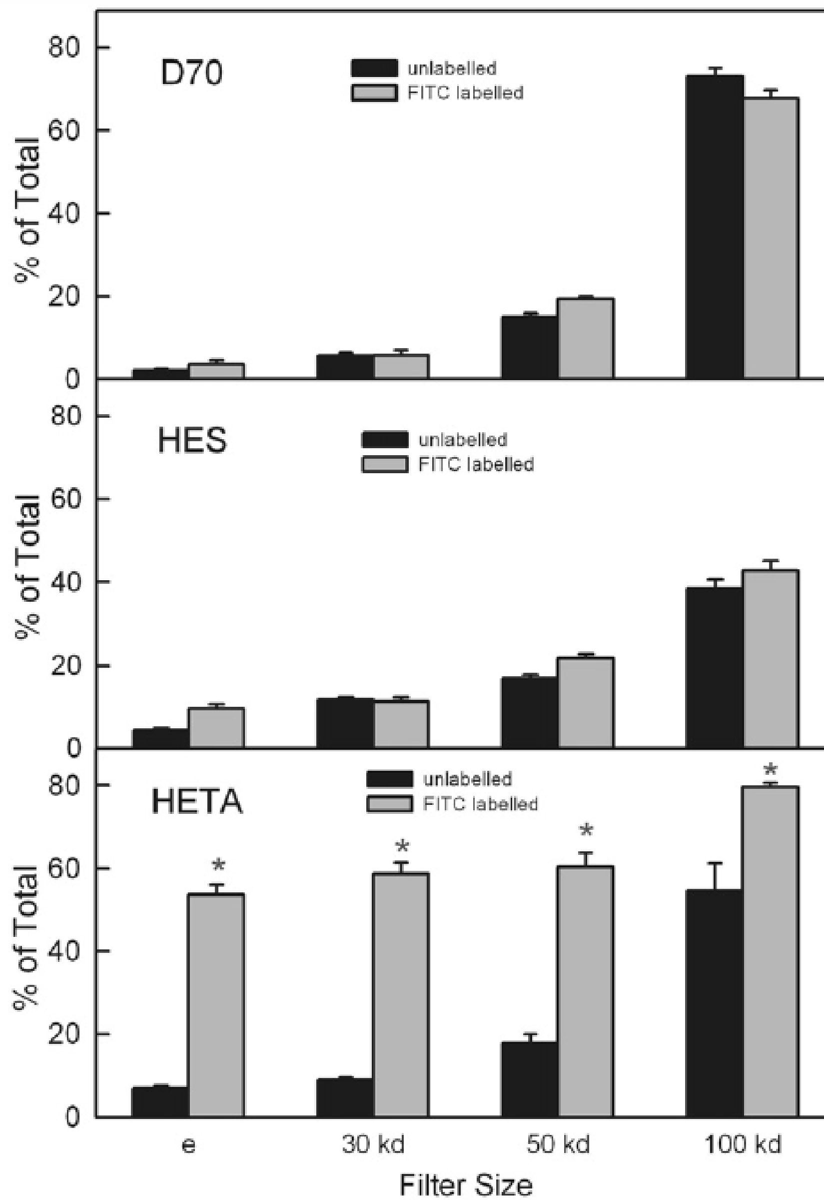

Figure 2-2. Effect of FITC-labeling on the Stability of Starches.
Pre-incubation (solid bars) and post-incubation (grey bars) samples of unlabeled and labeled Dextran, HES and UW-HES were assessed for starch degradation due to the labeling procedure with a series of molecular weight cut off filters. * -Indicates significant differences between pre- and post-incubation samples; $P<0.05$.

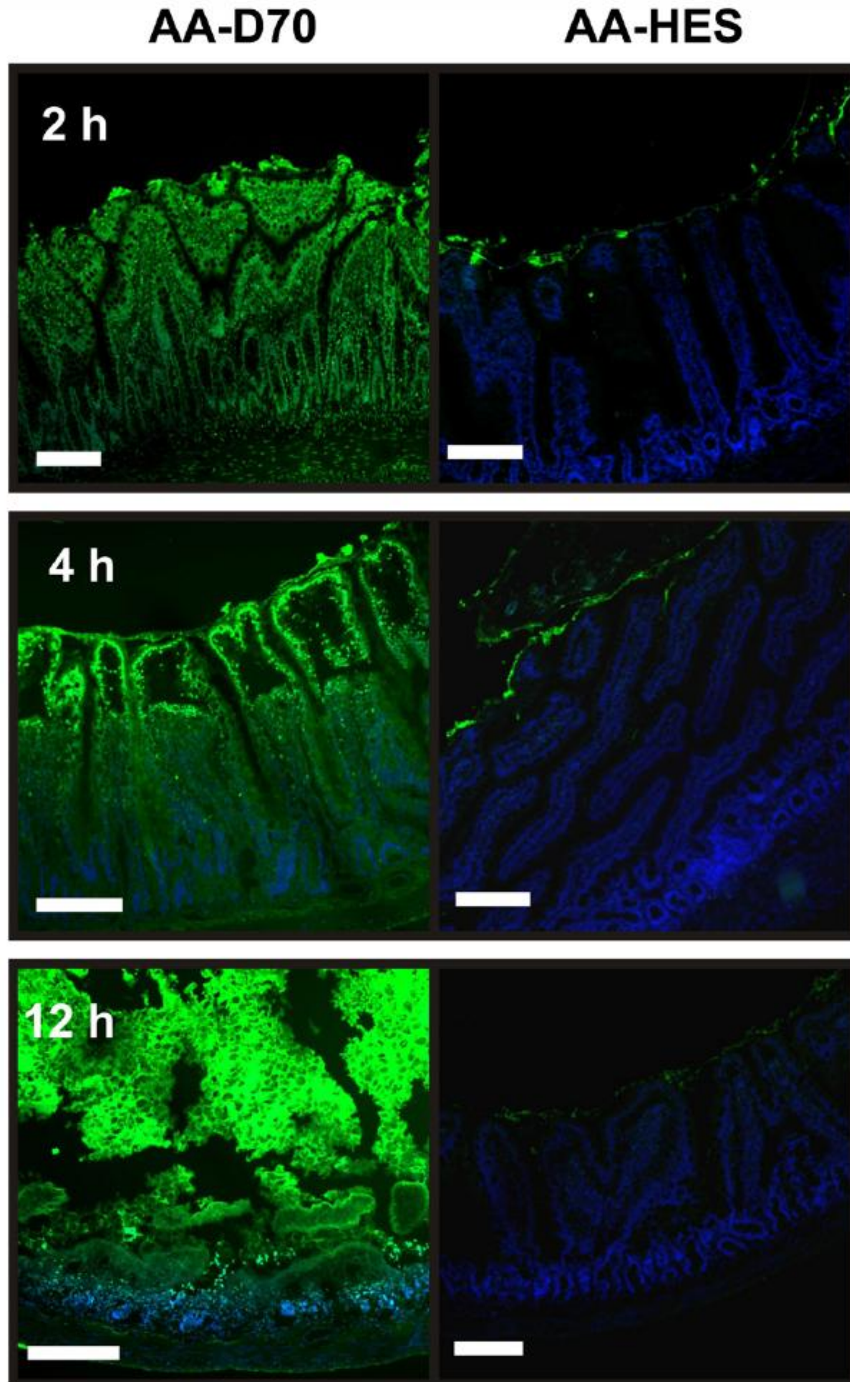

Figure 3-3. Mucosal Permeability of Dextran and HES during Cold Storage.
Fluorescence of intestinal tissues following 2, 4, 12 h cold storage with an Amino Acid-based (AA) preservation solution with dextran (D70) or hydroxyethylstarch (HES). Magnification was with 10x objective. Exposure times were standardized for all photographs. Standard wavelengths for excitation and emission for FITC and DAPI (for contrast purposes) were employed as described in the Methods section.

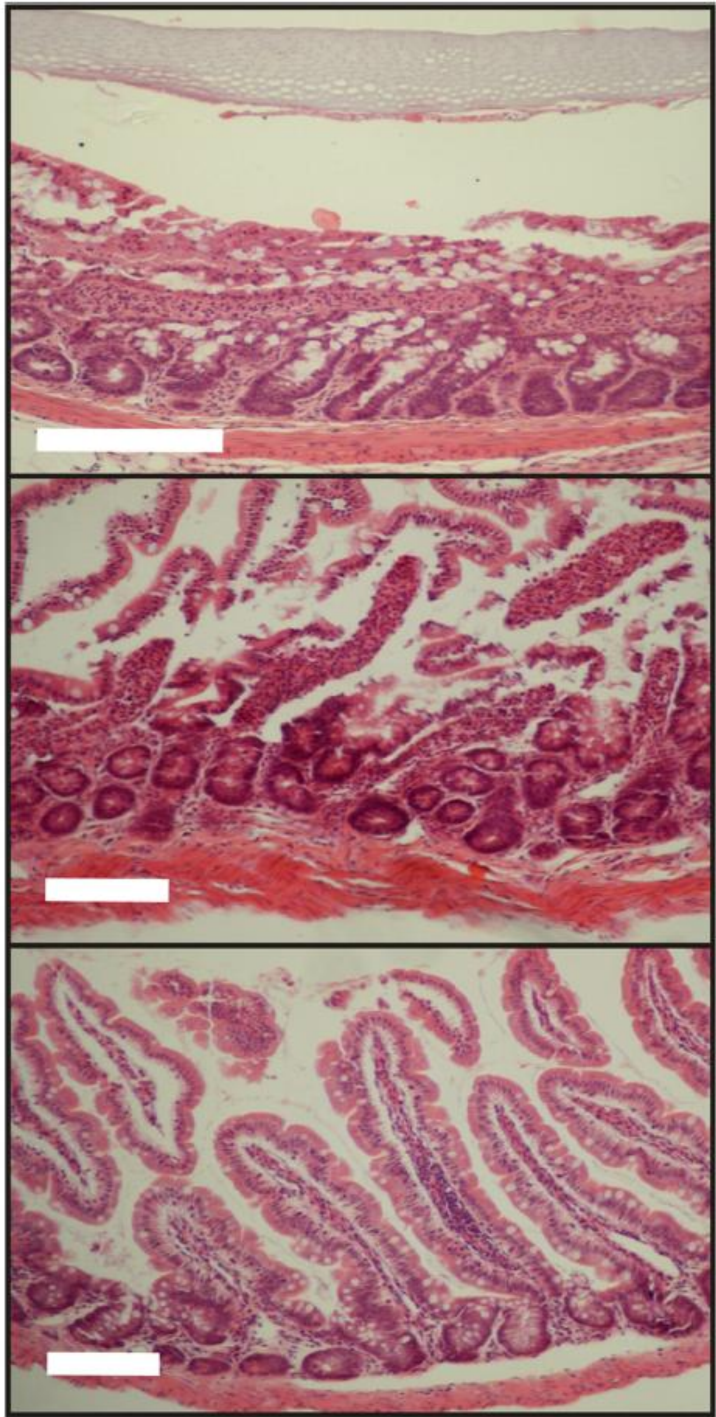

Figure 2-4. Histologic injury following 12h storage.
Photos of representative injury are presented as denoted in Table 1; injury depicted for AA-HES was significantly less than that incurred for UW or AA-D70; $P<0.05$. Grading of intestinal injury was based on a modified Park's classification of intestinal injury; details are as in Table 1.[17] Magnification was with 10X objective. Sections were 5 µm and were stained with Hematoxylin and Eosin.

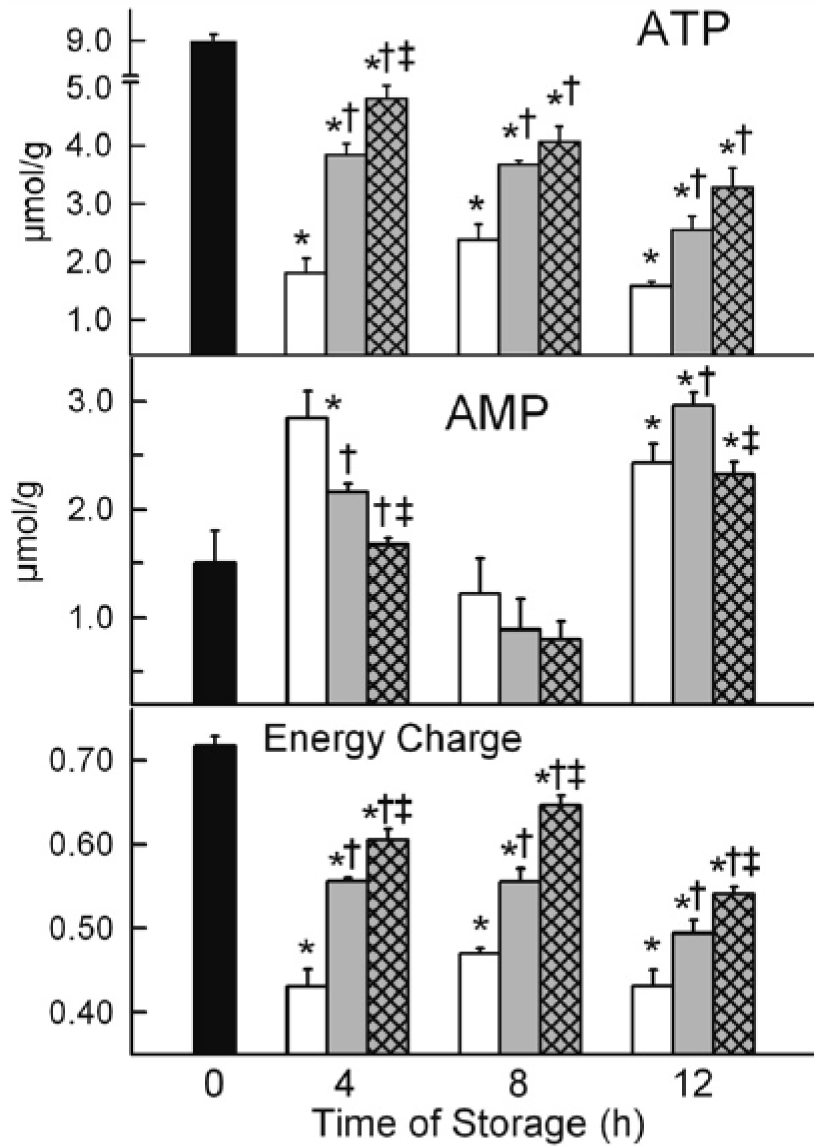

Figure 2-5. Effects of Dextran and HES on energy metabolism during cold storage. Data for ATP, AMP, and Energy Charge (EC) are presented. Energy charge was calculated by the equation: EC = (ATP+ADP/2)/(ATP+ADP+AMP). Group designations are: [white box], UW; [grey box], AA-D70; [cross-hatched box], AA-HES; [black box], freshly isolated. * -Significantly different from t=0 values; $P<0.05$. †, ‡ -Significantly different from UW and AA-D70 respectively; $P<0.05$.

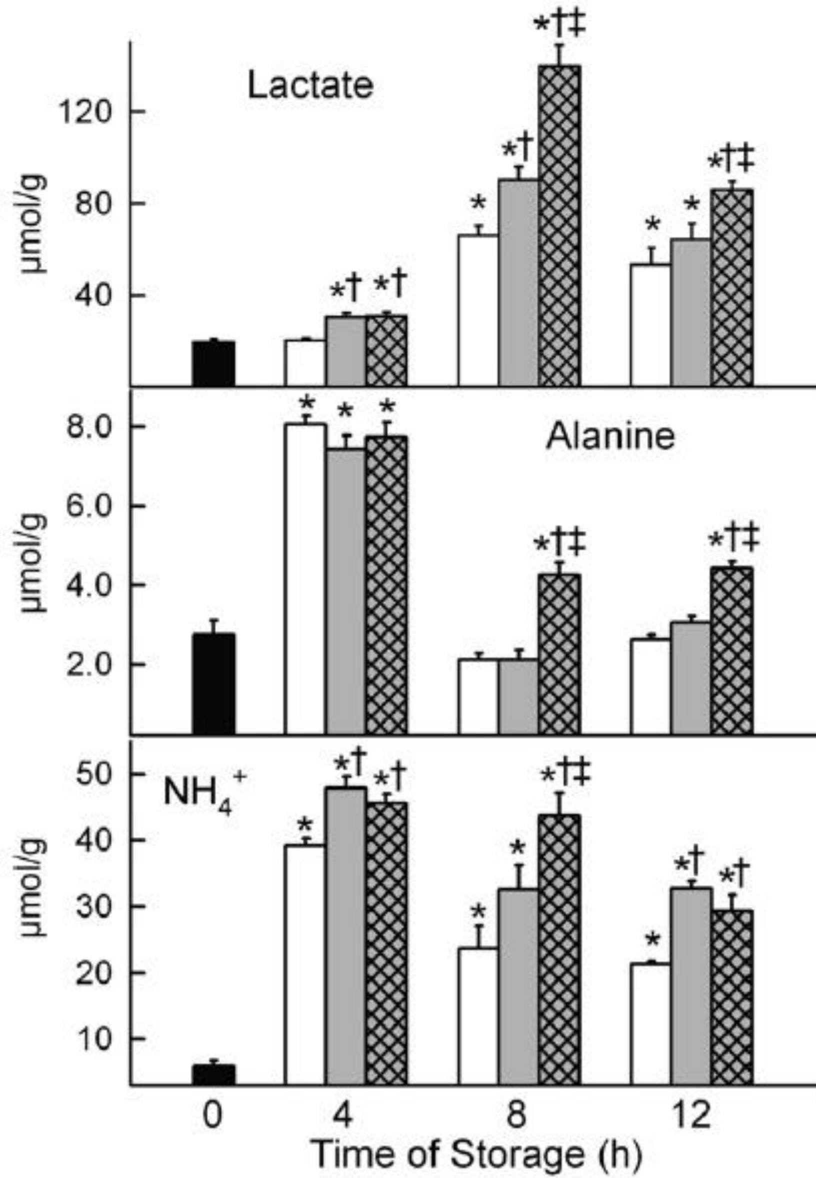

Figure 2-6. Effects of Dextran and HES on metabolic endproducts during cold storage. Data for lactate, alanine, and ammonium are presented. Group designations are: ▭, UW; ▭, AA-D70; ▭ , AA-HES; ▬ , freshly isolated. * -Significantly different from t=0 values; $P<0.05$. †, ‡ -Significantly different from UW and AA-D70 respectively; $P<0.05$.

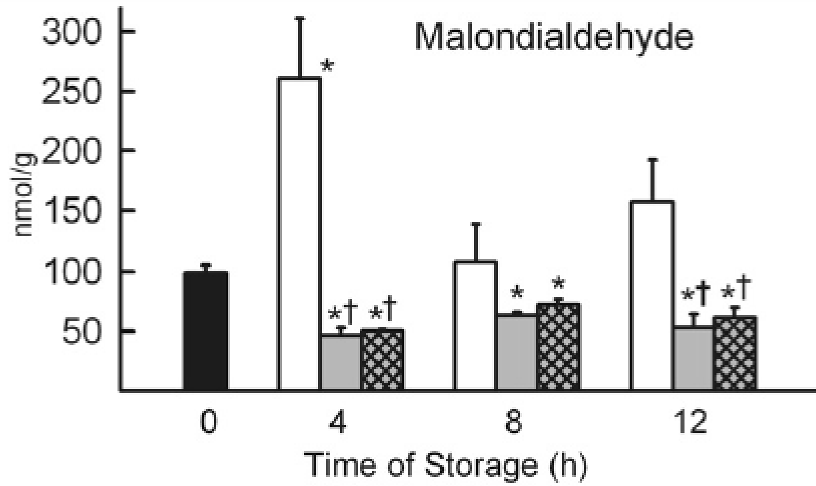

Figure 2-7. Effects of Dextran and HES on oxidative stress during cold storage. Data for malondialdehyde (MDA) are presented. Group designations are: [white box], UW; [grey box], AA-D70; [crosshatched box], AA-HES; [black box], freshly isolated.
* -Significantly different from t=0 values; P<0.05. †, ‡ -Significantly different from UW and AA-D70 respectively; P<0.05.

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Chapter 3: Optimizing the concentration of hydroxyethylstarch in an intraluminal preservation solution tailored to the intestine[2]

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Introduction

Small bowel transplantation is the only treatment available for patients with intestinal failure who have developed life-threatening complications or do not succeed with parenteral nutrition (1). However, long-term post-transplant mortality remains high (2). The mucosal layer of the intestine is highly sensitive to even brief periods of ischemia (3), resulting in progressive loss of barrier function as storage time prior to the actual transplant increases. Without a fully functional epithelial barrier, sepsis can occur due to bacterial translocation in the transplant recipient (4, 5). Although the mainstay of vascular preservation solutions, University of Wisconsin (UW) solution, has greatly extended safe storage times for the kidney, liver, and pancreas, it has not proven effective for the intestine; similar or improved results have been obtained using simple saline solutions (6, 7). Therefore, the development of novel preservation solutions must focus on graft integrity following clinically relevant storage times.

During the last several years, our laboratory has developed an amino acid (AA)-based preservation solution tailored to the metabolic requirements of the small bowel. Numerous *in vitro* and *in vivo* studies have demonstrated that luminal administration of

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this solution results in improved morphology and metabolic status of the small bowel following cold storage, ischemia-reperfusion, and experimental transplantation (8-12). Although the composition and route of administration differ between our solution and the UW solution, there exists a major commonality: the presence of osmotic impermeants to prevent tissue edema. At a molecular level, under normal circumstances, Na+/K+ ATPase ensures Na+ extrusion from the cell, which results in Na+ functioning as an osmotic agent outside the cell (13). The extracellular Na+ counteracts the osmotic pressure developed from intracellular proteins and impermeant anions. But during organ storage conditions, the situation is much different. Hypothermia and declining ATP levels collectively suppress Na+/K+ pump activity, causing Na+ and Cl- to follow their concentration gradient into the cell. As these ions enter, water follows and the cell swells. To offset the flow of water into the cell, an impermeant agent is required; 110-140 mmol/l has been established as the effective concentration range for an intravascular solution (13).

Hydroxyethyl starch (HES), a modified natural polymer of amylopectin (14), is the colloid contained in UW solution for oncotic support (15). Although the solution contains HES on the basis of preventing cold-storage interstitial edema (15), there exists some controversy over its effectiveness, especially for static organ storage. Some studies report that HES may be omitted from the UW solution without detrimental effects on organ preservation (16-18), while others indicate the protective role HES has on stored tissues (13, 15, 19-22). However, because these studies deal solely with the intravascular delivered UW solution, they do not provide information about the role of colloids in preservation solutions administered intraluminally for intestinal grafts.

In the previous chapter, we compared graft injury between tissues stored with AA solution containing either 70 kDa dextran or high molecular weight HES (2200 kDa) as the oncotic agent. Results indicated that tissues in the HES group exhibited superior energetic status and morphology following cold storage. However, an appropriate concentration of HES within the AA solution has yet to be established.

The current study tested the hypothesis that there is an optimal concentration of HES that will control osmotic stress during cold storage. Metabolic and morphological parameters of the intestine were assessed over a 24h time-course in groups receiving AA preservation solution supplemented with 0%, 2.5%, 5%, or 10% HES.

Materials and Methods

Summary of Experimental Design: Briefly, small intestine from rats was flushed intravascularly with University of Wisconsin (UW) solution, isolated and flushed intraluminally with a preservation solution which contained varying amounts (0-10%) of hydroxyethylstarch (HES). Intestines were stored at 4^oC and samples were taken over a 24h time-course.

Details: Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as organ donors. All experiments were conducted in accordance with Canadian Council on Animal Care policies.

Surgical Procedure and Organ Procurement: Rats were fasted 10-12 h and provided water *ad libitum*. Rats were induced with pentobarbital (65mg/250g; IP), followed by inhalational isoflurane (0.5-2%) to maintain anesthesia. Following a midline laparotomy,

the aorta was exposed infrarenally and at the celiac trunk. The supraceliac aorta was clamped and 2-4 ml modified University of Wisconsin (UW) solution was administered via the infrarenal aorta. The vena cava was transected to facilitate the outflow of blood and perfusate. The entire jejunum and ileum was subsequently harvested. On the 'back table', a nutrient-rich preservation solution, termed the "AA solution" to denote the high content of amino acids, was used to flush the lumen (40 ml; ~2.0 ml/g) allowing the effluent to exit uninhibited. Each end was ligated with 3-0 silk, leaving the bowel filled without turgor. The bowel was stored in 30 ml of each solution and stored on ice at 4°C. Tissue samples (1-2 g) were taken at 4, 8, 12 and 24 h post-flush. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and stored at –65°C until processed.

Experimental Groups: All experimental groups (n=4) were pre-treated luminally with AA-solution containing the following concentrations of HES: Group 1 – 0.0 %

Group 2 – 2.5 %

Group 3 – 5.0 %

Group 4 – 10.0 %

Composition of Solutions: AA solution contained: 0-10% Hydroxyethyl starch (HES), according to group designation, plus the following components: lactobionate (20 mM), adenosine (5mM), allopurinol (1mM), BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (15mM), glutamine (35mM), glucose/glutamate/aspartate (20mMeach), arginine/glycine/valine/asparagine/lysine/ threonine/serine (10Mm each), methionine/ornithine/leucine/isoleucine/histidine/proline/cysteine (5 mM each),

tyrosine/ tryptophan (1 mM each), hydroxybutyrate (3 mM), pH 7.4. Hydroxyethyl Starch was purified as previously described from 2-hydroxyethylstarch (HES), Aldrich #465143, 25000 cps] (23); the final HES product had a Mw = 2,200,000 daltons. Modified UW solution contained: lactobionic acid (100 mM), raffinose (30 mM), KOH (100mM), NaOH (15 mM), KH_2PO_4 (25 mM), $MgSO_4$ (5 mM), adenosine (5 mM), allopurinol (1mM), dextran (67.3 kDa; 5%), pH 7.4.

Histology: Full-thickness biopsies were formalin-fixed and processed to H&E sections. Ischemic injury was assessed using Park's grading system (24); Table 3-1.

Scanning Electron Microscopy: Specimens were glutaraldehyde-fixed and post-fixed with osmium tetroxide. Samples were dehydrated in ethanol and dried in a CO_2 critical-point dryer and mounted. Samples were sputter-coated with gold and examined under the Hitachi SEM S-2500 scanning electron microscope (25).

Sample Preparation and Metabolite Assay: Frozen samples were extracted 1:5 w/v in perchloric acid containing 1mM EDTA. Precipitated protein was removed by centrifugation (20 min, 20,000xg). Acid extracts were neutralized with 3M KOH/ 0.4M Tris/0.3M KCl and recentrifuged (20min, 14,000xg). Neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (26). Spectrophotometric analysis was performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ATP/ADP, total adenylates, lactate, and alanine. Values are reported as µmol per gram protein (27). Malondialdehyde was assessed from

frozen tissue homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA (28).

Statistical Analysis: Metabolite data were reported as mean ± SE for each group. Statistical differences between groups were determined using Analysis of Variance (ANOVA), followed by Tukey's *post hoc* test; $p<0.05$ was reported. Differences in histology grades was assessed by a non-parametric Kruskal-Wallis test, $p<0.05$ was reported.

Results

Histology (Table 3-1, Figure 3-1a, 3-1b, and3- 2)

Assessment of H&E sections revealed there were no significant differences in Parks grades between any group after 4h storage; median grades for Groups 1-4 were 3, 3, 1.5, and 0, respectively (Table 3-1). However, after 8 h, Groups 2 (2.5% HES) and 3 (5% HES) showed significantly less tissue damage than Group 1 (0% HES); median grades for Groups 2 and 3 were 2.5 (indicating moderate subepithelial clefting), while Group 1 had a median grade = 7 (transmucosal infarction), $P<0.05$. Group 4 (10% HES) grades were highly variable, ranging from 0-8 with a median of 2.5. However, after 12 h the high degree of variability in Group 4 dropped dramatically, consistently revealing significant injury compared to Groups 2 and 3; both Groups 3 & 4 exhibited significantly greater injury with grades of 7 & 8 compared to grades of 3 for both Groups 2 & 3 ($P<0.05$). After 24 h storage, only Group 3 (median grade = 3) revealed significantly less tissue damage than Group 1 (median grade = 7.5, $P<0.05$) or Group 4 (median grade = 7, $P<0.05$). Group 2 had a median grade = 5, which was not significantly different than any

of the other groups including Group 3 due to high variability. Interestingly, the range of injury in Group 2 was grades 3-8 with the majority of specimens (4/6) having considerable villus damage including loss of villus tissue; whereas in Group 3, 4/6 specimens only had minor issues of subepithelial clefting.

Scanning Electron Microscopy (SEM) is a powerful tool and can be useful as corroborative evidence in conjunction with standard light microscopy. In Figure 3-2, micrographs of representative injury were presented for tissues subjected to 12 h cold storage. Micrographs of Groups 1 and 4 showed a high degree of damage at the intestinal villi, with extensive epithelial villus denudation and infarction of regenerative cryptal regions. Contrasting these observations, despite equivalent Parks' grades = 3 in both Groups 2 and 3, only Group 2 exhibited consistent epithelial sloughing at villi apices (an early step in the process of villus denudation). No evidence of a loss of epithelial integrity was apparent in Group 3 tissues exhibiting a median injury grade = 3.

Energetics (Figure 3-3)

ATP levels exhibited a progressive decline in all experimental groups over 24 h storage. However, ATP in Group 4 were generally lowest of the four experimental groups, followed by Group 1. Of particular relevance was the superior maintenance of ATP in Group 3; values were at least 0.6-1.0 μmol/g greater than in the other groups following 8-12 h, $P<0.05$.

ATP/ADP ratios are a reflection of the mitochondrial enzyme machinery to generate ATP via oxidative phosphorylation. ATP/ADP ratios exhibited trends similar to ATP, with Group 3 exhibiting the highest values of all groups over the first 12 h storage ($P<0.05$ after 8-12h compared to all groups).

Alterations in total adenylate (TA) levels were similar to ATP. Although Group 4 showed poorer maintenance of the TA pool ($P<0.05$), Group 3 consistently demonstrated superior maintenance of TA compared to Group 1; $P<0.05$. Between 8-24h, the optimal concentration of hydroxyethylstarch with respect to maintenance of TA was 5% (Group 3; $P<0.05$).

End-Products (Figure 3-4)

Evidence of metabolic stress was apparent in all experimental groups. There was an accumulation of lactate over the first 8 h storage (highest levels were detected in Groups 2-4 with increases of 84±1 µmol/g, $P<0.05$); Group 1 increased by 68 µmol/g (80% of the other groups). By 12-24 h levels exhibited a progressive decline, suggesting a loss of accumulated anaerobic end-product from the tissue.

Amino acid metabolism plays a major role in intestinal metabolism and alanine is typically produced as a result of various transamination reactions as other amino acids enter the Kreb's cycle. Thus alanine accumulation in isolated intestine is a positive indicator of amino acid catabolism as a potential energy source during cold storage. At all time-points, Group 3 exhibited the highest amount of alanine production ($P<0.05$ compared to all groups at all time-points; this was particularly pronounced at 24 h.

Oxidative stress (Figure 3-5)

The generation of Malondialdehyde (MDA), a by-product of lipid peroxidation, did not occur until after 4h storage. By 8h, Groups 1 and 2 exhibited significant increases compared to Groups 3 and 4 ($P<0.05$); these increases continued until 12h at which point peak values of 200 ± 4 nmol/g had been reached. Although MDA levels did

increase in Groups 3 and 4 following 12 h storage (to 163 μmol/g for both groups), levels were almost 20% lower than the control group (Group 1). Interestingly, values in Group 4 (containing the highest percentage of starch) dropped significantly lower than Group 3 (and the other groups) after 24h, $P<0.05$.

Discussion

Throughout the last three decades, the major clinical application of hydroxyethylstarch (HES) has been for plasma volume expansion. HES has documented safety for use in humans; compared to other colloids such as gelatins, dextrans, and albumin, the frequency of anaphylactic reactions due to HES is significantly lower (14). Recognizing its colloidal properties, Belzer and Southard included HES in their UW organ preservation solution to control tissue edema (13). The molecular weight of HES is a major determinate of its colloidal activity. Although the HES in UW-solution has a weight average Mw of 250,000 Da with a fraction of starch molecules as low as 100,000 Da (29), we selected HES with a Mw of 2.2 million Da for our preservation solution in this study. The permeability characteristics between vascular endothelial cells and intestinal epithelial cells are necessarily different. Permeability of the intestinal lumen has a much greater Mw limit than the vascular endothelium; hence, a significantly higher Mw starch is needed for use in our luminally administered preservation solution than the starch used in the intravascularly delivered UW-solution. Hydrostatic and impermeant forces govern the movement of fluids between intracellular and extracellular compartments. However, hydrostatic forces do not apply to the maintenance of fluid movement in this study. The model of graft storage presented in this report is that of static storage; thus, only impermeant forces contribute to net flux. To date, damaging shifts in net fluid flow

during cold-storage are suggested to originate from the intestinal vasculature and/or lumen (30, 31). By administering a common UW vascular flush to all treatment groups, cold-storage fluid shifts of vascular origin were controlled for. Thus, solely altering HES concentration in the intraluminal AA solution allowed us to attribute any inter-group differences in mucosal morphology and biochemical parameters to fluid shifts of luminal origin. Presuming that the presence of an oncotic agent is a necessary determinant affecting static cold storage when employing an intraluminal preservation solution, one would expect that alterations in morphology of the mucosal epithelium, along with tissue energetics are concentration-dependent.

In the current communication, we were able to determine the net consequences of varying starch concentrations in our nutrient-rich preservation solution with standard light microscopy, corroborated with scanning electron micrographs; although we did not directly assess real-time osmotically-induced alterations in morphology. Interestingly, the two extremes in AA solution HES concentration (0% and 10%) had the most detrimental effects on tissue morphology out of all concentrations tested. Clearly, the requirement for osmotic or oncotic agents has an optimal level as with many biological processes and deviation from this optimum results in hypotonic or hypertonic stresses. In this study, the negative effects of 10% HES were likely attributable to a generated hypertonic environment, thereby drawing cellular fluids into the intestinal lumen and resulting in osmotic stress with secondary effects on energy-producing processes. The consequence of 0% HES was similar, except that the initiating event was a hypotonic luminal environment that caused the epithelial cells to swell and possibly lyse. Again, secondary negative effects on energy production exacerbated the insult. Both 2.5% and 5% HES exhibited favourable morphology throughout most of the

storage time-course. 2.5% HES-treated tissue exhibited good maintenance of mucosal morphology, with morphological grades equivalent to 5% HES-treated tissues after 8 and 12h; however, electron micrographs showed evidence of apical epithelial sloughing potentially suggesting the initiation of villus denudation, the result of a non-optimal HES concentration. Overall, both H&E sections and electron micrographs indicated that 5% HES-treated tissue displayed superior mucosal morphology throughout the 24h storage time-course. In this treatment group, no evidence of irreversible damage to the epithelium or to the mucosa (ie. Crypt infarction) was evident. This concentration was the only one to have a significantly lower Park's grade median than 0% (control group) after 8, 12, and 24h storage times. Moreover, the 5% group had the least variation in Park's grading thus reflecting a consistent and reproducible effect. As expected, groups containing the highest (10%) and lowest (0%) concentration of HES incurred the most mucosal damage during storage. This trend was seen after 8h storage, and most evident after 12 h. In the 0% and 10% groups after 12h, scanning electron micrographs showed direct visual evidence of complete villus denudation with consistent infarction of the regenerative cryptal regions.

The ability of mucosal epithelial cells to maintain their barrier function directly depends on energy levels (32). Tight junctions between cells, which are responsible for the barrier, consist of dynamic, energy-requiring proteins (8). Cellular energy levels are quickly exhausted during ischemic storage because many energy-consuming processes are still functioning even at hypothermia (33). As graft energetics decline, the mucosal epithelium becomes less capable of maintaining its barrier function (34). More than any other solid organ, intestinal graft quality reflects energy levels such as, ATP and TA. Tight junction dilation is linked to increases in transepithelial flux of enteric bacteria,

ultimately leading to sepsis (3, 35, 36). Fortunately, tight junction perturbation is reversible; repletion of ATP levels results in tight junction re-assembly and restored epithelial barrier function (9). In order for ATP levels to efficiently regenerate upon blood flow reestablishment and reperfusion, purine levels must be maintained in a phosphorylated form (ATP, ADP, or AMP). Also, ATP-ADP ratios are usually used as a direct measure of the integrity of mitochondrial oxidative phosphorylation (8). Hence, maintenance of cellular energetics typically indicates superior overall preservation of graft morphology and function.

In the present study, increasing concentrations of HES in our luminal AA preservation solution had variable effects on tissue energetics. Although at each concentration tested (0-10% HES) all groups experienced a progressive decline in ATP over increasing storage times, 5% HES was the most effective concentration, maintaining higher ATP levels at each storage time point. Furthermore, this improvement maintained significance at the more clinically relevant storage times of 8 and 12 hours; additionally there was superior preservation of the TA pool (after 8h, 12h, 24h). In addition to a greater potential for ATP regeneration following storage, higher TA levels reflect lower rates of purine catabolism, potentially yielding hypoxanthine. This is particularly beneficial since hypoxanthine oxidation results in the production of a reactive oxygen species (ROS) in the form of superoxide. Like ATP, ATP-to-ADP ratios also declined in all groups throughout cold storage. However, at 8-12 h storage, 5% HES in AA solution also demonstrated improved maintenance of this energy parameter. Evidence of increased amino acid metabolism with 5% HES was observed in heightened levels of alanine, the primary by-product of intestinal amino acid metabolism; conversely in 0, 2.5 and 10% HES-treated tissues, lower end-product levels were

reflective of reduced ATP-generating catabolism. Additionally, only 0% HES treatment resulted in a differential reduction in lactate, reflecting a negative influence of hyposmotic stress on glycolytic energy production.

Previously, our laboratory showed that oxidative stress is an important component of static organ storage as a result of increased oxidative metabolism in the context of an intraluminal preservation strategy (8, 37). Malondialdehyde (MDA) is a by-product of lipid peroxidation and levels are a reliable index of oxidative stress. Cell membranes contain polyunsaturated fatty acids that are vulnerable to oxidative attack and broken down to yield the peroxidation product, MDA (38). Membrane lipid peroxidation, in addition to declining ATP levels, are both key factors responsible for the disruption of mucosal epithelial cell tight-junctions (39), contributing to loss of barrier integrity and subsequent enteric bacterial translocation and sepsis. In our study, 5% HES had the lowest amounts of MDA after 8 and 12h storage. This effect was most striking at 8h, as MDA levels with 5% HES were significantly lower than with 0% and 2.5% HES. Interestingly after 24 h MDA was significantly lower with 10% HES suggesting a potential concentration-dependant anti-oxidant effect due to HES. However, direct assessment of antioxidant capacity of HES is required for definitive proof of any proposed antioxidant properties.

In this study, we focused on events pertaining directly to the static cold storage of intestinal tissue; the events of transplantation necessarily provide an additional level of complexity in that ischemia-reperfusion can exacerbate any injurious events incurred during storage. Although it is beyond the scope of the present study, several starch-related effects relating to the inhibition of inflammatory processes will clearly become important following reperfusion after transplantation of the graft. HES may exert a

protective effect on the intestinal mucosa by influencing deleterious inflammatory processes activated by oxidative stress incurred during ischemia and subsequent reperfusion. Superoxide molecules, a consequence of the xanthine oxidase-mediated oxidation of hypoxanthine, result in the accumulation and activation of neutrophils (40). Both recruited neutrophils and oxygen free radicals activate the transcription factor NF-κB (41). In turn, NF-κB may lead to the expression of specific genes involved in the production of mediator synthesis and amplification of the inflammatory response (42). Upon adherence, activated neutrophils release multiple degradative enzymes (myeloperoxidase, elastase, collagenase) which potentiate the cell damage initiated by ischemia (43, 44). Therefore, any event reducing the involvement of neutrophils in IR injury would likely improve functioning of the transplanted graft. Several investigators have focused on the relationship between HES and NF-κB. In a model of lung capillary permeability in endotoxic rats, Tian et al. (45) proposed a mechanism for HES inhibition of NF-κB. In their study, HES reduced LPS-induced activation of NF-κB in neutrophils and the lungs, leading to decreased microvascular endothelial permeability. Other reports have also demonstrated that HES exerts an anti-inflammatory effect via the inhibition of NF-κB activation (46). In the intestine, NF-κB activation triggers endogenous proinflammatory mediators during IR injury (41). When NF-κB activation is inhibited, the degree of tissue injury diminished, which can be accompanied by a reduction in neutrophil recruitment (41). In the current study, it is plausible that a significant amount of HES could penetrate the intestinal mucosa and cross the basolateral membrane into the capillaries. Although high MW HES (2.2 million Da) was used in our study, it is an average weight, containing a small number of molecules in the low MW range (14), as does the UW solution (39). Moreover, due to the low degree of substitution, large HES

molecules may be hydrolyzed into smaller fragments by endogenous amylases, thereby permitting small amounts of HES leaking from the lumen into the intestinal capillaries; this may be beneficial in an ischemia-reperfusion situation such as that of stored organs following transplantation. However this causal relationship remains to be clarified in the realm of intestinal preservation and transplantation.

In conclusion, different concentrations of HES in our nutrient-rich preservation solution had variable effects on ischemic cold-storage of small bowel in rodents. Tissue energetics, oxidative stress, and histology supported the concept that in our recently developed nutrient-rich intraluminal preservation solution, an HES concentration of 5% is optimal for superior graft preservation during static cold storage. The use of oncotic rich intraluminal solutions for graft preservation provides a novel approach to preventing the short and long term morbidity related to intestinal transplantation.

Tables and Figures

Grade	*Description*
0	Normal mucosa
1	Subepithelial space at villus tip
2	More extended subepithelial space
3	Epithelial lifting along villus sides
4	Denuded villi
5	Loss of villus tissue
6	Crypt layer infarction
7	Transmucosal infarction
8	Transmural infarction

Table 3-1. Microscopic Criteria in the Grading of Intestinal Tissue Injury.
Adapted from Park et al. (24)

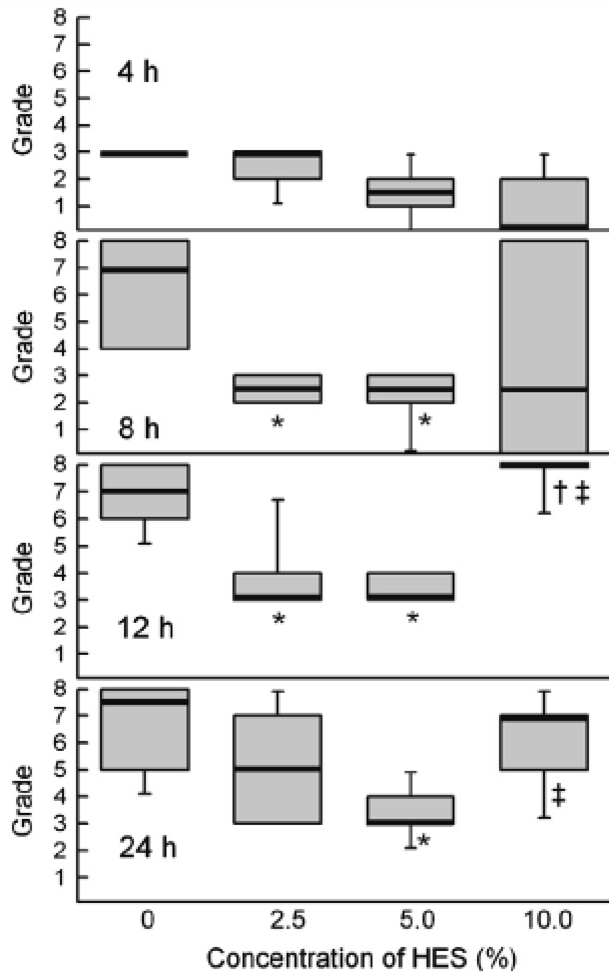

Figure 3-1a. Effect of HES on intestinal morphology.
Histology grades were assigned as in Table 1. Thick lines within the boxes indicate median values; box limits indicate 1st and 3rd quartiles; error bars indicate 95% confidence intervals. *, †, ‡ -Significantly different from 0%, 2.5% and 5% HES, respectively; $P<0.05$.

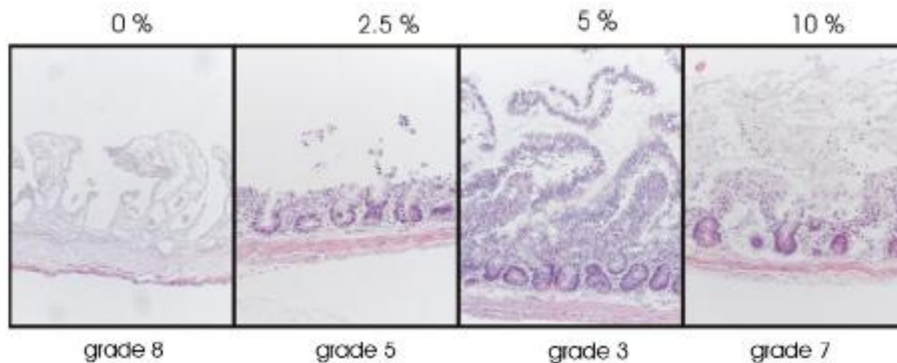

Figure 3-1b. Mucosal histology after 24h storage.
Grades represent median values. 10X objective.

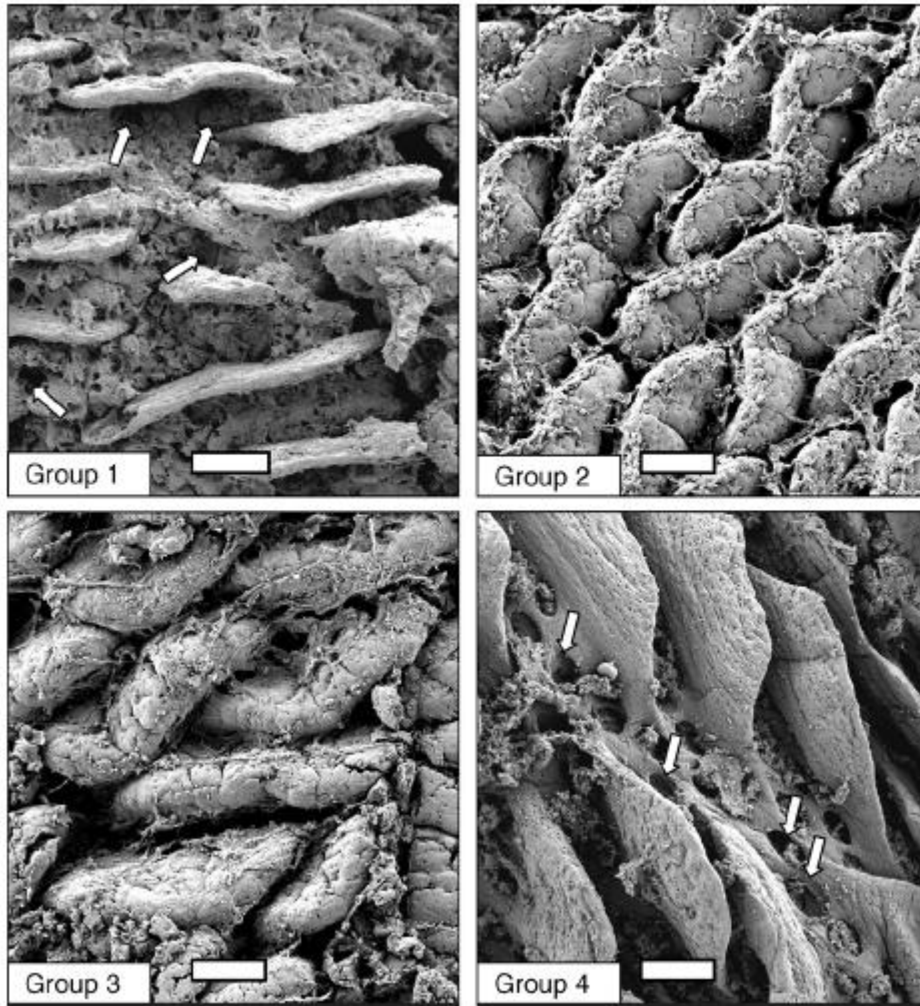

Figure 3-2. Scanning Electron Micrographs of intestine following 12h cold storage. Magnifications were 80-150X and were representative. Arrows in Groups 1 and 4 photos indicate areas of cryptal infarction.

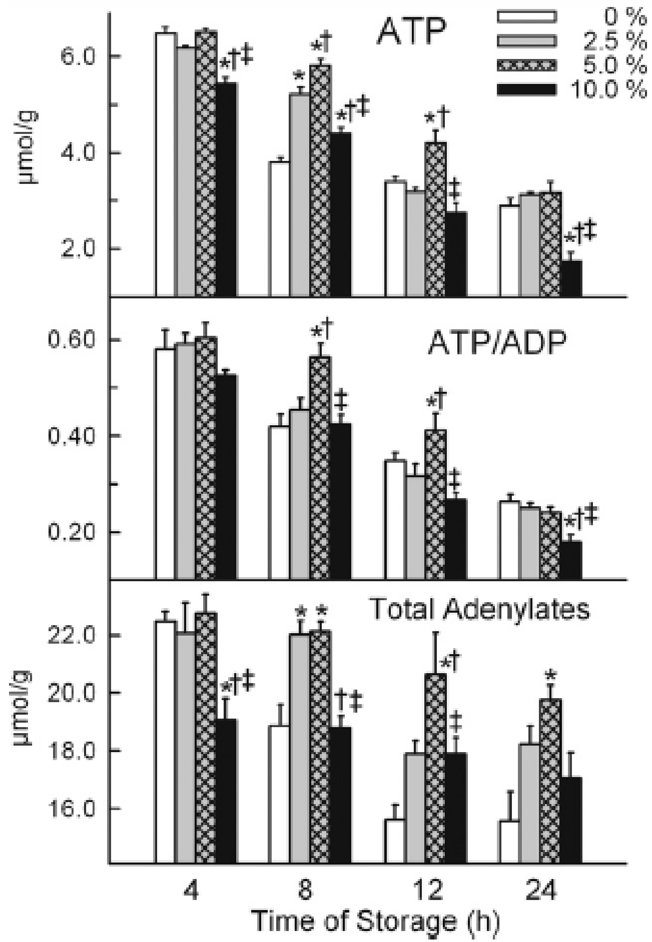

Figure 3-3. Effect of HES on tissue energetics: ATP, ATP/ADP, Total Adenylates. Group designations are: [white box], Group 1; [gray box], Group 2; [hatched box], Group 3; [black box], Group 4. *, †, ‡ -Significantly different from 0%, 2.5% and 5% HES, respectively; $P<0.05$.

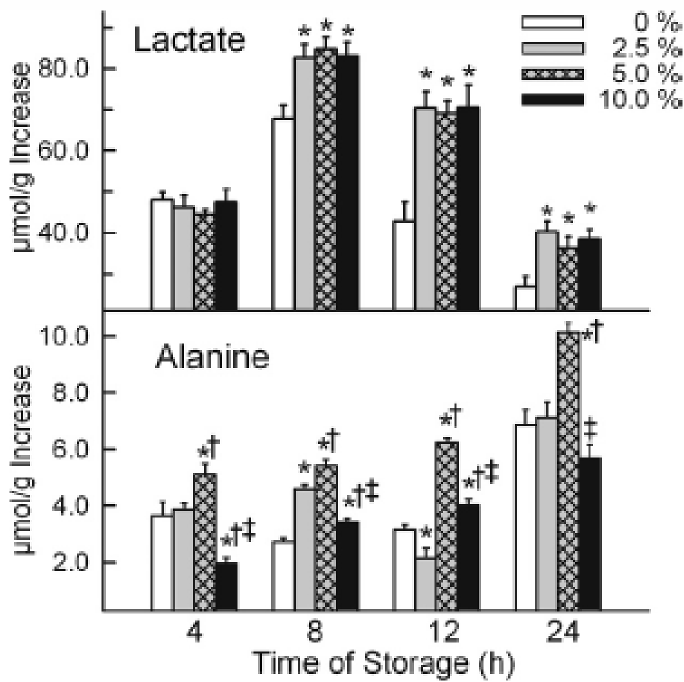

Figure 3-4. Effect of HES on metabolic end products.
Group designations are: ▭, Group 1; ▭, Group 2; ▭, Group 3; ▬, Group 4. *, †, ‡ -Significantly different from 0%, 2.5% and 5% HES, respectively; $P<0.05$.

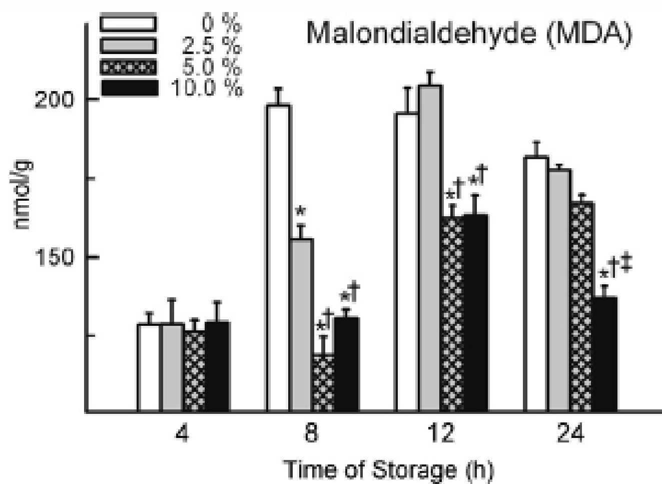

Figure 3-5. Effect of HES on malondialdehyde, an index of oxidative stress.
Group designations are: ▭, Group 1; ▭, Group 2; ▭, Group 3; ▬, Group 4. *, †, ‡ -Significantly different from 0%, 2.5% and 5% HES, respectively; $P<0.05$.

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Chapter 4: General Discussion and Conclusions

In the mid to late 1980s, Belzer and Southard developed the University of Wisconsin (UW) preservation solution. To date, the UW solution has been the most significant advance in organ preservation technology, extending storage times, and improving graft and patient survival rates (1). Even though the UW solution has been in use for over 20 years, it still remains the 'gold standard' solution for static organ preservation of the liver, kidney, and pancreas (2). One should note, however, that Histidine-Tryptophan- Ketoglutarate (HTK) solution has been in widespread use for intra-abdominal organs since the early 90s in Europe, and has become increasingly utilized in North America since 2002 (2). In terms of intestinal preservation though, there is no single solution that has proven truly effective (3).

Currently, the small intestine is harvested from the donor as part of a multi-organ procurement, involving a common intra-aortic flush with the preservation solution employed by the specific institution (most commonly UW solution). Flushing the vasculature both cools the organs to inhibit enzymatic activity, and facilitates later reperfusion (4). Since it would not make practical sense to interfere with this clinically established vascular preservation method, our laboratory has developed a novel approach to small bowel preservation, involving an intraluminal preservation solution tailored to the metabolic requirements of the small intestine. By administering our solution to the lumen of the intestine, osmotic agents, buffers, antioxidants, and substrates are delivered to the luminal surface of enterocyts. This solution is termed AA (amino acid) solution to denote the large amino acid component. It has been under development throughout the last decade. The benefits of delivering our intraluminal AA

solution, in addition to the routine UW vascular flush, have been proven in both large and small animal studies (5,6).

Similar to other preservations solutions, the AA solution contains energy substrates, buffers, and antioxidants. Also like other solutions, the AA solution utilizes osmotic agents to control damaging fluid shifts that occur during cold storage.

When discussing fluid balance during intestinal storage, there are some important considerations to keep in mind. The observation that damaging fluid shifts arise from both intraluminal and intravascular compartments (7) dictates that the AA solution must control fluid flux during the preservation period. One strategy to prevent osmotic stress involves supplementing the AA solution with cell impermeant molecules, which creates a force that counteracts the movement of water from the intestinal lumen into the tissue. In order for a molecule to function as an effective impermeant, however, it must remain within the intestinal lumen throughout storage. Although dextran 70 (70 kDa) does not enter systemic circulation or enterocytes after orogastric administration in healthy rats (8), the properties of the intestinal barrier during ischemic cold storage are altered. Tight junctions dependent on ATP are responsible for maintaining intestinal barrier function, and they dilate in response to declining energy levels (9). Molecules that do not penetrate the healthy intestinal barrier can potentially do so during storage.

Early formulations of the AA solution included dextran between 65 and 70 kDa as the impermeant agent (6,10,11). Hydroxyethylstarch (2,200 kDa) has replaced dextran in more recent versions of the AA solution (5,12). The reasoning behind the switch largely related to the idea that a larger molecular weight molecule may be required in order to remain within the intestinal lumen during ischemic cold storage.

However, the effectiveness of either agent has never been compared. Dextran and HES are similar in many ways, both being colloids and playing a role in volume expansion, but the molecules in the studies presented here differ substantially in regards to MW. Dextran 70 has a MW of 70 kDa, whereas HES has a MW of 2,200 kDal. Molecular weight is the major determinate of a colloid's osmotic activity *in vitro* (13).

There were two primary goals of the research presented in the current communication: 1) determine which molecule, D70 or HES, could more effectively control osmotic stress during ischemic cold storage of the small intestine; 2) define the optimal concentration of the superior agent. In chapter 2, fluorescent microscopy revealed that FITC-labeled D70 easily penetrated the intestinal barrier after only 2 hours of cold storage and continued to do so throughout the entire time course of 12 hours. Contrasting this result, FITC-labeled HES remained captive within the intestinal lumen at 2 and 4 hours, with trace amounts entering the tissue after 12 hours. Considering that an oncotic agent must reside within the lumen over the entire storage period to remain effective, these results suggested that the AA solution containing HES should possess a superior ability to control osmotic stress. Suspicions were confirmed after comparing energy levels and histologic injury. Compared to the UW and AA-D70 groups, intestines in the HES group had higher levels of ATP and energy charge, and lower AMP levels at each time point. Histology showed that grafts preserved with AA solution containing HES had significantly less damage at 4 and 12 hours compared to the UW and AA-D70 groups. Together, these results allowed us to conclude that HES is an effective oncotic agent for use in our intraluminal, nutrient-rich preservation solution, while D70 is not.

The study presented in chapter 3 focused on defining the optimal concentration of HES within the AA solution. Tissues were flushed and stored with AA solution

supplemented with 0, 2.5, 5, or 10% HES. Overall, the 5% group showed superior maintenance of ATP, ATP/ADP, and TA levels, a finding most pronounced at 8 and 12 hours. The 5% group also managed to maintain energy levels to some degree, albeit to a lesser extent than the 5% group. The extremes in concentration (0 and 10%) performed the most poorly. Histological assessment revealed a similar trend. The lowest levels of graft injury were found in the 5 and 2.5% groups, with the 5% performing slightly better at 4 and 24 hours post-storage. Again, the 0 and 10% groups fared the worst, especially at 12 and 24 hours. In an unexpected result, tissues treated with 10% HES had the lowest levels of oxidative stress overall, as measured by malondialdehyde (MDA). Although out of the scope of the study, one possible explanation for the low MDA levels in the 10% HES group is that HES may have some inherent antioxidant properties. Based on these outcomes, we concluded that 5% is the optimal concentration of HES for use in the AA solution. However, the impact of these results extends beyond the initial goal of determining a suitable HES concentration. For the first time, the necessity of an oncotic agent for intraluminal preservation of the small intestine has been proven.

Limitations and Future Directions

The two studies presented within the current communication employed an *in vitro* cold storage model of intestinal preservation in the rat. Similar models have been utilized by our lab in the past, and as demonstrated here, they continue to function as a valuable first step when optimizing specific components within the AA solution. Tissue energy levels, oxidative stress, and architecture are evaluated following storage, and give clues about graft viability. With that said, *in vitro* experiments are limited in that

they cannot provide direct information regarding graft function after the reintroduction of oxygenated blood.

Although much more technically difficult, an orthotopic intestinal transplant model in the rat represents a logical next step in the evaluation of impermeant support within the AA solution. Such an experiment would provide clinically relevant endpoints, such as recipient response and survival. Using syngeneic Lewis rats would eliminate rejection considerations, allowing us to attribute any differences in outcome between groups to the preservation conditions. An extension of the cold storage, *in vitro* studies presented here, two groups of tissues would undergo cold storage in AA solution containing either D70 or HES. Also, addition of another group containing an impermeant molecule not previously tested in our laboratory would create an interesting dimension to the experiment. Intraluminal delivery of polyethylene glycol (PEG 3350) during hypothermic intestinal preservation has recently proven beneficial (14, 15). The idea of supplementing the AA solution with PEG 3350 and comparing its effects to D70 and HES in the context of a storage/transplantation model is intriguing.

Moving from a small animal model of storage and subsequent transplantation in the rat, large animal experiments using pigs would represent the intermediate step before moving to human tissues. Given that the permeability characteristics of the rat and pig are not identical, initial experimentation with pigs should aim to re-optimize osmotic support in the AA solution to porcine tissues. It is likely that a larger molecular weight molecule is required for the AA solution to effectively control damaging fluid shifts during storage of pig intestine than the HES proven effective for rat tissues. Again, after optimizing impermeant support through cold storage studies, a more clinically relevant storage/transplantation study should be undertaken. A positive result in such

an endeavour would warrant the time and effort involved in obtaining human tissues for further investigations.

Akin to the situation when switching from rat to pig tissues, the human intestinal barrier differs from that of the pig, and so *in vitro* experiments would be required to ensure that impermeant support within the AA solution is optimized for human tissue. Following this step, the notion of taking the AA solution to clinical trial may potentially be considered; however, before such a monumental task is undertaken, all of the components within the AA solution must also be optimized to achieve the most positive results.

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